LITERATURE REVIEW
1.0 DIURETICS

Diuretics are traditionally defined as substances that increase the amount of urine i.e. of fluid excreted by the kidney. However, diuretics elevate the amount of urinary sodium and subsequently that of water i.e. they are basically natriuretic. Substances that increase urinary water selectively, all of which are under investigation are called aquaretics. Diuretics augment natriuresis by impeding the reabsorption of sodium that has filtered through glomerulus. The sodium reabsorptive process takes place at different part of renal tubular system, through different mechanism. About 65% of filtered sodium (Na) is reabsorbed in the proximal convoluted tubule, about 25% at the level of thick ascending limb of loop of Henle. Between 7-9% is reabsorbed in the early distal tubule and about 2% of filtered sodium leaves the nephronal tubular fluid in the connecting tubule and the cortical collecting duct.

Diuretics are widely used in cardiovascular medicine. They are currently recommended as one of the options for the first line treatment of uncomplicated essential hypertension. They are formally indicated in hypertension accompanied by congestive heart failure and/or renal insufficiency and are obligatory in all forms of congestive heart failure unless definitely contraindicated (Reyes and Taylor, 1999).

Although various agents that increase urine flow has been described since antiquity, it was not until 1957, with the synthesis of chlorothiazide, that practical and powerful diuretic agent became available for wide spread use. Thus, the science of diuretics is relatively new (Ives, 1997).

2.0 PHARMACOLOGICAL CLASSIFICATION AND RENAL ACTIONS OF DIURETICS

The diuretic agents have been variously classified according to their chemical structures (Table 1), the potency of their effects on sodium transport (Table 2) as well as their mechanisms and sites of action in the
nephron (Table 3). Since the chemical classification of diuretics is of little medical use and less satisfactory, the most acceptable classification is based on the transport mechanisms at each of the four major electrolyte transport sites in the nephron, which diuretics are active at each nephron segment and how the impairment of sodium reabsorption at each locus affects diuretic potency. The four major loci of electrolyte reabsorption throughout the nephron are illustrated in fig. LR 1.

Modern diuretics currently used in cardiovascular medicine include three classes of substances (Reyes and Taylor, 1999). Some diuretics drugs have their main site of renal action in the early distal tubule (Velazquez, 1987), other compounds impede sodium reabsorption principally in the loop of Henle (Greger and Wangemann, 1987) and the third class of modern diuretics inhibit the passage of sodium from the nephronal tubular contents to the interstitial fluid in the connecting tubule and in the cortical collecting duct (Horisberger and Giebisch, 1987). An older group of substances, which have their main site of renal actions in the proximal convoluted tubule and act by inhibiting the enzyme carbonic anhydrase, constitutes a fourth class of available diuretics. These drugs exert a mild natriuretic action and various undesirable effects; therefore, their use in cardiovascular therapy should always be transient and restricted to special circumstances in patient presenting congestive heart failure (Reyes and Taylor, 1999).

2.1 Proximal tubule (site 1)

Carbonic anhydrase inhibitors, of which acetazolamide is the most conspicuous representative, impede bicarbonate reabsorption in the proximal convoluted tubule. Given that the part of the sodium reabsorption that takes place at this level is linked to the reabsorption of bicarbonate, carbonic anhydrase inhibition results in increased natriuresis, which is accompanied by renal retention of hydrogen ion and its attendant metabolic acidosis, and by a large increase in urinary potassium excretion (Presig et al., 1987).
Several diuretic agents have secondary rather than primary effects in the proximal nephron, including furosemide, bumetanide, piretanide, chlorthiazide, metolazone and indapamide.

2.2 Medullary ascending limb of loop of Henle (site 2)

Loop diuretics interfere with the linked reabsorption of sodium, chloride, and potassium that takes place in the thick ascending limb of loop of Henle (Greger and Wangemann, 1987). This action results in increases in natriuresis, chloruresis and diuresis. This response to loop diuretics is accompanied by increased urinary excretions of potassium, calcium and hydrogen ion, and thereby a rise in plasma pH. The natriuretic effects of loop diuretics increases as a function of the dose over a wider dose range than the urinary sodium outputs that occurs in response to all other diuretic classes. For this reason, loop diuretics are said to be "high ceiling diuretics". They are capable of causing excretion into urine of more than 15-20% of filtered sodium. They have proved especially effective in patients with reduced glomerular filtration rates, as the thiazide group of drugs is often ineffective in these patients (Reubi and Cottier, 1961).

2.3 Early distal convoluted tubule (site 3)

Early distal tubular diuretics reach the tubular lumen through an organic acid excretory pathway that operates in the cells of proximal convoluted tubule and then reach to cells of the early distal tubule. There they impede the reabsorption of sodium (Velaquez, 1987). The natriuretic action of early distal tubular diuretics increases kaliuresis and hydrogen ion excretion and hence plasma pH shifts towards higher values. These substances lower the amount of calcium that is excreted in urine.

2.4 Late distal convoluted tubule and collecting duct (site 4)

Late distal convoluted tubule and collecting duct diuretics have their site of renal action at the connecting tubule and mainly at the adjacent cortical collecting duct. At these levels, sodium is reabsorbed
from the tubular fluid in exchange for potassium and hydrogen, which are excreted into the tubular lumen. This physiological process is stimulated by aldosterone (Horisberger and Giebisch, 1987). Since they conserve potassium and hydrogen ion, therefore they are called potassium and hydrogen retaining diuretics. Frequently they are mentioned as "Potassium sparing diuretics". This expression should be avoided for at least two reasons. First, when these drugs are considered as diuretic or natriuretic agents, their effect on urinary potassium and proton excretion should be qualified consistently and not for what these agent do when they are co-prescribed with a thiazide type or a loop diuretic. Second, potassium and hydrogen retaining diuretics may cause hyperkalemia, a serious complication not implied by the term "Potassium sparing" (Reyes and Taylor, 1999). Some potassium and hydrogen retaining diuretics act by blocking a sodium channel involved in this exchange e.g. Amiloride* and Triamterene*. While others are antialdosterone agents. Spironolactone**is the prototype of these diuretic. They are generally co-prescribed with an early distal tubular or a loop diuretic, insofar as their use as a single diuretic may result in hyperkalemia.
Fig. LR 1: Diagrammatic representation of the nephron showing the four sites of solute reabsorption.
Table 1: Chemical Classification of diuretics

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Chemical nucleus</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Heterocyclic sulphonamides</td>
<td>Acetazolamide, Methazolamide</td>
</tr>
<tr>
<td>2.</td>
<td>Benzothiadiazine (Thiazides)</td>
<td>Chlorthiazide, Hydrochlorothiazide, Benzthiazide</td>
</tr>
<tr>
<td>3.</td>
<td>5-sulfamoyl benzoic acid derivatives</td>
<td>Furosemide, Bumetanide</td>
</tr>
<tr>
<td>4.</td>
<td>4-amino-3-pyridine sulfonylemides</td>
<td>Torasemide</td>
</tr>
<tr>
<td>5.</td>
<td>Phenoxy acetic acid</td>
<td>Ethacrynic acid</td>
</tr>
<tr>
<td>6.</td>
<td>2,4,7-Triamino-6-arylpteridines</td>
<td>Triamterene</td>
</tr>
<tr>
<td>7.</td>
<td>Pyrazinoylguanidines</td>
<td>Amiloride</td>
</tr>
<tr>
<td>8.</td>
<td>Steroid nucleus, containing</td>
<td>Spironolactone</td>
</tr>
<tr>
<td>9.</td>
<td>Mercurial diuretics</td>
<td>Chloromerodin, Meralluride, Mersaly</td>
</tr>
<tr>
<td>10.</td>
<td>Miscellaneous</td>
<td></td>
</tr>
<tr>
<td></td>
<td>i) Hexahydroxy alcohols</td>
<td>Mannitol</td>
</tr>
<tr>
<td></td>
<td>ii) Xanthine bases</td>
<td>Theophylline</td>
</tr>
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</table>

Table 2: Differential pharmacodynamic features of the diuretic classes in use

<table>
<thead>
<tr>
<th>Diuretic class</th>
<th>Effect on the urinary excretion of</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Potassium</td>
</tr>
<tr>
<td>Carbonic anhydrase inhibitors</td>
<td>Increase</td>
</tr>
<tr>
<td>Loop diuretics</td>
<td>Increase</td>
</tr>
<tr>
<td>Distal tubular diuretics</td>
<td>Increase</td>
</tr>
<tr>
<td>Potassium and hydrogen retaining diuretics</td>
<td>Decrease</td>
</tr>
</tbody>
</table>
Table 3: Diuretic substances in use

<table>
<thead>
<tr>
<th>Class</th>
<th>Main site of renal action</th>
<th>Prototype substance</th>
<th>Other substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cabonie anhydrase inhibitors</td>
<td>Proximal convoluted tubule</td>
<td>Acetazolamide</td>
<td></td>
</tr>
<tr>
<td>Loop diuretics</td>
<td>Thick ascending limb of loop of Henle</td>
<td>Frusemide</td>
<td>Azoseinide, Bumetanide, Etharycrynic acid, Pirtenamide, Torasemide</td>
</tr>
<tr>
<td>Early listed tubular diuretics</td>
<td>Early distal tubule</td>
<td>Hydrochlorothiazide</td>
<td>Chlorthaldone, Cicletanine, Clopamide, Clorexolone, Indapamide, Metolazone, Thiazides, Tienilic acid, Xipamide</td>
</tr>
<tr>
<td>Potassium and hydrogen retain-ing diuretics</td>
<td>Connecting tubule and cortical collecting duct</td>
<td>Spironolactone*, Amiloride**</td>
<td>Potassium canrenoate, Triamterene</td>
</tr>
</tbody>
</table>
3.0 FUNCTIONAL STATE OF THE NEPHRON AND DIURETICS DOSE RESPONSE

Diuretic therapy should be based on pathophysiological considerations, which take into account the absorption, and excretion capacities of the different segments of the nephron in the different disease syndrome for which these drugs are administered.

3.1 Dose-response effects of diuretics

Correlation of the urinary concentration of a diuretic with the simultaneous fractional excretion of sodium produces a characteristic sigmoid relationship (Brater et al., 1980; Brater et al., 1986). The plateau of the curve indicates the intrinsic maximal activity of the diuretic. The effective disposition of a diuretic may be characterized by relating the amount excreted in the urine to the glomerular filtration rate.

In the syndrome with a normal nephron population but where the effective arterial blood volume is reduced, for example heart failure, hepatic cirrhosis, and nephrosis, the dose response relationship is much flatter. This implies that the intrinsic excretory ability of functioning nephrons is reduced by hyperreabsorption in the proximal tubule and by the decrease in sodium load delivered to the distal segment. This limits the efficacy of high doses of diuretics, with the result that increasing the dose of diuretic produces no further increase in diuresis. In contrast, in chronic renal failure, the intrinsic nephron excretory capacity is relatively increased. This is related to rejection of sodium in the distal tubule with an increase in its fractional excretion. Furthermore, the fractional excretion of sodium is doubled with each 50% decrease of glomerular filtration rate, the so called 'magnification phenomenon' (Knauf and Mutscher, 1994). The changes in the dose efficacy curve are not related to changes in function of tubular system; they reflect the response to the amount of sodium available in the respective nephron segment, that is, the site of action of the diuretic.
3.2 Clinical application of the concept of intrinsic diuretic activity

The concept of the intrinsic activity of a diuretic suggests that the most effective therapeutic avenue to consider is blockade of electrolyte reabsorption in different nephron segments with a combination of different diuretics, rather than confining therapy to high doses of a single drug. This is likely to be most effective in clinical conditions where arterial blood volume is reduced and hence manipulation of electrolyte transport in the proximal nephron allows control of the function of more distal segment e.g. in congestive heart failure and monotherapy with a loop diuretic, the maximum effectiveness of the diuretic is rapidly reached as the dose is increased. When a proximal tubular diuretic, such as acetazolamide is co-administered however, sodium excretion is further enhanced.

4.0 DIURETIC RESISTANCE

When a diuretic drug is administered, the rate of urinary sodium and chloride excretion usually increases above baseline leading to a period of negative sodium and chloride balance. This natriuresis and chloriuresis is the hallmark of effective therapy. Yet, within several days to weeks, net daily solute and water losses decline and eventually approach pre diuretic levels, despite continuous drug administration. These changes in diuretic responsiveness result from adaptive processes that occur during diuretic therapy. When these processes become manifest i.e. once the desired extracellular fluid (ECF) volume has been attained, they are clinically useful and prevent progressive ECF volume contraction. When these processes develop prior to achieving the desired ECF volume, they can be viewed as contributing to diuretic resistance (Ellison, 1999).

Resistance to loop diuretic is a common clinical phenomenon. Virtually all patients with edematous disorder manifest some element of diuretic resistance (Brater, 1994). The determinations of normal responses to loop diuretics may be divided into pharmacokinetic and
pharmacodynamic components. In both components, the key element is the amount of diuretic reaching the urine.

4.1 Pharmacokinetic determinants

The pharmacokinetic determinants of response to a loop diuretic are function of both the total amount of diuretic reaching the urinary site of action and also the time course of delivery into urine (Kaojaren et al., 1982; Brater, 1989). The amount of diuretic reaching the urine is determined by its concentration in serum and its renal clearance. Serum concentration in turn is determined by the dose, bioavailability, and volume of distribution of the diuretics. With all diuretics, there is dose proportionality in terms of the area under the serum concentration time curve. This translates to dose proportionality for delivery of drug into urine.

The pharmacokinetic determinants of resistance to a diuretic require assessment of whether or not the disease process affects the circulating concentration of the diuretics and whether its renal clearance is changed. It has been demonstrated that in rats devoid of albumin where there is no protein binding to hold the loop diuretic in the circulation, the volume of distribution is very large. As a result, serum concentration is less than the drug to be delivered to the kidney and into urine. The clinical implication of this role of protein binding and volume of distribution are evident in patients with nephrotic syndrome (Inoue et al., 1987). In addition to the total amount of drug appearing in the urine, the time course of delivery of diuretic into the urine is also an important determinant of loop diuretic response (Kaojaren et al., 1982). An explanation for this phenomena is that persistance of diuretic at the site of action at concentration in an effective region of the concentration response curve will produce a greater overall effect as compared with exposure of the active site to the same total amount of diuretic, but during which a substantial component of the time of exposure is at concentration that are suboptimal. Hence the effect of a dose of diuretic
administered at a continuous rate that delivers effective concentration to the site of action will be greater than that of the same dose administered as a single bolus during which effective concentration quickly dissipate to those which are insufficient to block sodium reabsorption (Meyel et al., 1994).

Loop diuretics that are currently available have short half-lives with the exception of torasemide. It is possible that longer half-life of torasemide could impart a greater overall response than would occur with a comparably potent dose of a short acting diuretic. Another option is that slow release formulations would result in a greater overall response.

4.2 Pharmacodynamic Determinants

The pharmacodynamic determinants of resistance to loop diuretics are poorly elucidated. The decline in solute and water losses after the therapy of diuretics, which could be limited to several days to weeks, to prediuretic level is considered to be because of certain adaptive processes that occur during diuretic therapy. These diuretic adaptations can be classified as (Ellison, 1999):

➢ Those that occur during diuretic action.
➢ Those that cause sodium retention in the short term (causing diuretic NaCl retention).
➢ Those that increase sodium retention chronically (Braking phenomena).

4.2.1 Immediate adaptation of diuretics

All diuretic drugs act by inhibiting sodium entry pathways in the apical membranes of renal tubule epithelial cells. Since apical sodium transport pathways are nephron-segment specific. Hence, each class of diuretics inhibits sodium transport predominantly along a specific segment of nephron. When the sodium chloride (NaCl) reabsorption along the thick ascending limb is inhibited by loop diuretics, the NaCl concentration in fluid that enters the distal tubule is greatly increased.
In a study, the sodium concentration in fluid entering the distal tubule of rats rose from 42 to 140 millimolar (mM) during acute loop diuretic infusion (Hropot et al., 1985). Sodium absorption along the distal tubule increased by more than 100% because NaCl transport varies directly with luminal NaCl concentration. The bulk of increase in NaCl transport along the distal tubule appears to traverse the thiazide sensitive NaCl co-transporter. In another experiment, in microperfused rat, distal tubules, raising the luminal NaCl concentration two fold, increased the transepithelial sodium transport by a factor of 3; this increase could be blocked entirely by chlorothiazide (Wright et al., 1982). This type of adaptation occurs during the period of diuretic-induced natriuresis. The net effect of acute diuretic administration on urinary Na and Cl excretion reflects the sum of effects in the diuretic-sensitive segment (inhibition of NaCl reabsorption) and in diuretic insensitive segments (secondary stimulation of NaCl reabsorption). The importance of compensatory processes to blunt acute effects of diuretics is exemplified by carbonic anhydrase inhibitors, which inhibit Na transport across the proximal tubule (Wright, 1982).

4.2.2 Short-term adaptations to diuretics

The half-life of most diuretic (especially the loop diuretics) is relatively short. Thus, serum diuretic concentration is often below the natriuretic threshold during a portion of each day, except when the drugs are infused constantly. The second type of adaptive response to diuretic administration occurs after the peak natriuresis has occurred and is most prominent when the drug concentration in plasma and tubular fluid declines below the diuretic threshold. In this situation, the diuretic is no longer present in the tubule fluid to inhibit renal sodium reabsorption and period of NaCl retention; often termed as 'post diuretic NaCl retention' begins. The net effect of the diuretic during 24 hours, therefore, reflects a period of natriuresis (when NaCl transport is
inhibited by the diuretic) and a period of antinatriuresis (when the drug concentration is low, before the next dose is given) (Ellison, 1999).

Mechanisms that contribute to post diuretic NaCl retention have been investigated and grouped into 3 classes:
1) Factors that result from changes in ECF volume
2) Factors that result from diuretic induced increases in distal sodium, chloride and fluid delivery
3) Factors that result from direct effects of diuretic drugs on tubule transport process.

Important contributors to ECF volume-dependent NaCl retention are:
1) Changes in the glomerular filtration rate
2) Activation of renin/angiotensin/aldosterone system
3) Stimulation of efferent renal sympathetic nerves
4) Suppression of atrial natriuretic peptide secretion
5) Suppression of renal prostaglandin secretions

Thus, changes in ECF volume play a central role and the post diuretic NaCl retention can be prevented by administering sodium, potassium, and chloride at rate sufficient to equal diuretic induced losses (Almeshari et al., 1993).

The renin/angiotensin/aldosterone axis contributes importantly to renal NaCl homeostasis, but evidence for an important role of these hormones in post diuretic NaCl retention has been conflicting. In normal volunteers, post-diuretic NaCl retention was unaffected by the angiotensin converting enzyme inhibitor, captopril, given in doses sufficient to block furosemide induced changes in angiotensin II and aldosterone levels (Kelly et al., 1983). Furthermore, diuretic induced changes in blood pressure were similar with or without captopril, suggesting that hypotension did not mediate the sodium chloride retention in angiotension-converting enzyme (ACE) inhibitor group. This indicates that post-diuretic NaCl retention can occur without activation
of the renin/angiotensin/aldosterone system. However, they do not indicate that stimulation of the renin/angiotensin/aldosterone axis does not participate in post diuretic NaCl retention when it does occur.

Peterson et al., (1991) showed that systemic adrenergic α-1 receptor blockade attenuates the reduction in NaCl excretion that occurs during short-term furosemide-induced ECF volume depletion in rats. However, administration of prazosin in doses that block the pressor response to α adrenergic agonists does not prevent post diuretic NaCl-retention even when both prazosin and captopril are administered concurrently, to block both the renin/angiotensin/aldosterone axis and effects renal nerve activity normal post diuretic NaCl retention may occur.

Suppression of atrial natriuretic peptide secretions following the diuretic administration to both normal individuals and patients with nephrotic syndrome, chronic glomerulonephritis and essential hypertension is seen (Jesperssen et al., 1990). In some studies, atrial natriuretic peptide concentration has declined before significant changes in extracellular or blood volume occur. In these cases, it has been suggested that furosemide induced changes in venous capacitance may underlie this effect.

Despite the central role of ECF volume contraction in post diuretic sodium chloride retention, a volume independent component of adaptation also contributes to tendency toward sodium chloride retention that occurs after loop diuretic administration. One mechanism by which diuretic drugs may increase the tendency for sodium chloride retention directly without changes in ECF volume, involves diuretic induced activation of sodium ion transporter within the diuretic sensitive nephron segment. Both the absorptive and secretory isoforms of the loop diuretic sensitive Na-2k-2Cl cotransporter (NKCC) are activated
allosterically by decrease in intracellular chloride concentration (Payne et al., 1995).

Another mechanism by which diuretic drugs may enhance the tendency to NaCl retention involves stimulation of transport pathways in nephron segment that lie distal to the target of diuretic action. Post diuretic NaCl retention can have major effects on the clinical efficacy of diuretic drugs. If dietary NaCl intake is low, then diuretic, NaCl retention does not compensate for drug induce NaCl losses and NaCl balance become negative. If on other hand, dietary NaCl intake is high, post diuretic retention can compensate entirely for the initial NaCl losses and salt balance may be neutral, even from first day of diuretic therapy (Bosch et al., 1977; Wilcox et al., 1983) despite impressive increase in urine volume after each dose. Therefore, dietary NaCl intake is key determinant of diuretic efficacy, especially for the short acting loop diuretic (Ellison, 1999).

4.2.3 Chronic adaptation to diuretics

When diuretics reduce ECF volume effectively, NaCl balance gradually returns to neutral despite continued diuretic administration (Wilcox et al., 1983). This is called 'braking phenomena' and it occurs when the magnitude of natriuresis following each diuretic dose declines. Several factors, acting in concert, may participate in chronic adaptation. A critical factor that is necessary for the braking phenomena to occur is a decline in the ECF volume. They showed that the magnitude of the natriuretic response to diuretic declines on once daily furosemide treatment of humans consuming a low NaCl diet. In contrast, when dietary NaCl intake is high, ECF volume depletion does not occur and the magnitude of diuretic-induced natriuresis does not decline. Relative or absolute ECF volume contraction limits NaCl excretion by reducing the amount of NaCl that is filtered and by increasing the amount of NaCl that is reabsorbed.
In experimental animals, decline in renal blood flow occurs during chronic diuretic treatment, but decline in glomerular filtration rate are usually modest, unless volume depletion is extreme or renal perfusion is otherwise compromised. This is because renal blood flow decline proportionately more than glomerular filtration rate and ECF volume contraction increases the filtration fraction (glomerular filtration rate (GFR)/renal blood flow) (Ellison, 1999).

Many of the effector systems that participate in post diuretic NaCl retention also may participate in chronic adaptation to diuretic drugs. **Physical factors:** A rise in filtration fraction increases the protein oncotic pressure in peritubular capillaries (more protein free filtrate is formed/ml of blood flow thereby contracting the plasma volume around a constant amount of serum protein). The increased peritubular oncotic pressure increases solute and fluid reabsorption especially in the proximal tubule, where physical factor play a prominent role. ECF volume contraction also enhances proximal solute and fluid reabsorption by decreasing the renal interstitial pressure during chronic diuretic treatment.

**Sympathetic renal nerve activity:** Renal nerves may contribute to NaCl retention in edematous disorder and renal nerve activity is stimulated when furosemide is administered either to normal or volume depleted animals (Dibona and Sawin et al., 1985). However, experimental models of chronic diuretic administration have failed to substantiate a central role for renal nerve activity (Peterson and DiBona, 1992). Although it seems clear that renal nerves do not play a critical role in mediating compensation to chronic diuretic use in normal humans and animals, the consistent observation that diuretic do stimulate renal nerve activity suggest that renal nerves contribute to diuretic adaptation in some patients,

**Renin/angiotensin/aldosterone:** Diuretics stimulate renin secretion via several mechanisms:
Loop diuretics stimulate renin secretion by inhibiting NaCl uptake into macula densa cells by blocking Na-K-2Cl uptake. Blocking Na-K-2Cl uptake at the macula densa stimulates renin secretion directly leading to a volume-independent increase in angiotensin II and aldosterone secretion.

Loop diuretics stimulate renal production of prostacyclin. Cycloxygenase inhibitors inhibit the increase in renin secretion that results from loop diuretic administration, suggesting that the increased secretion of prostaglandins play a critical role in diuretic-induced renin release (Frolich et al., 1976).

ECF volume contraction stimulates renin secretion via vascular effects on juxtaglomerular cells.

Renal nerves directly stimulate renin secretion via interaction with β adrenergic receptors on juxtaglomerular cells that affects cellular production of cyclic adenosine monophosphate (cAMP).

ECF volume contraction inhibits secretion of atrial natriuretic peptide. Epithelial hypertrophy and hyperplasia: when a diuretic is administered, solute delivery to distal segments increases leading to load dependent increases in solute reabsorption. When solute delivery and solute reabsorption increases chronically, epithelial cell undergo both hypertrophy and hyperplasia. Infusion of furosemide into rats continuously for 7 days increased the percentage of renal cortical volume occupied by distal nephron cells. Distal convoluted tubule cell volume increased by nearly 100% with accompanying increased in luminal membrane area per length of tubule and in mitochondrial volume per cell (Kaiissling et al., 1985; Kaiissling et al., 1988). Chronic loop diuretic administration increases the Na/K ATPase activity in distal convoluted and cortical tubules (Scherzer et al., 1987; Wald et al., 1989) and increases the number of thiazide sensitive Na-Cl co-transporters, measured as the maximal number of binding site for [3H] metolazone. In
one study, chronic furosemide treatment increased expression of mRNA encoding the thiazide sensitive NaCl co-transporter (Obermuller et al., 1995). But in another study, no change was seen on furosemide infusion (Moreno et al., 1998). Compared with tubules from normal animals, tubules from animals treated chronically with loop diuretics absorb Na ion and chloride upto 3 times more rapidly (Ellison et al., 1989).

The diuretic-induced signals that initiate changes in distal nephron structure and function are poorly understood. Increased production of angiotensin and aldosterone secretion may contribute to hypertrophy and hyperplasia. Angiotensin is a potent mutagen; Angiotensin II receptors have not been localized definitely to distal convoluted tubule (DCT) but recent functional studies do suggest that DCT cells express angiotensin II receptors (Wang and Giebisch, 1995).

Another hypothesis is that cellular ion concentration regulates epithelial cell growth directly (Stanton and Kaissling, 1989). The hypothesis predicts that blockade of apical sodium entry would lead to atrophy of epithelial cells. Chronic treatment of rat with distal convoluted tubule reduces activity Na/K ATPase and Na transport capacity of DCT segments (Garg and Narang, 1987; Morsing et al., 1991) but other structural effects complicate these experiments. Recent experiments have shown that immune reactivity for insulin like growth factor-1 (IGF-1) and for and IGF-binding protein (IGFBP-1) increases during chronic treatment of rats with loop diuretics (Kobayashi et al., 1995). IGF-1 has been shown to participate in regeneration of injured or ischemic renal tissue and promote all differentiation and proliferation in vitro. Whether these changes in IGF expression mediate the effects of diuretic on distal nephron structure remains to be established.

Although experimental data concerning structural and functional responses of distal nephron to chronic treatment with diuretic drug come predominantly from studies employing experimental animal, Loon et al., (1989) reported that chronic treatment with furosemide for 1 month,
there is enhancement of sodium excretion significantly from a dose of thiazide diuretic. They estimated transport capacity of the DCT as the portion of Na and Cl reabsorption that could be inhibited by thiazide diuretic. Although this data was indirect but consistent with data derived from experimental animal given loop diuretic chronically.

5.0 DIURETIC RESISTANCE IN COMMON CLINICAL CONDITIONS

5.1 Congestive heart failure

Most of the symptoms of congestive heart failure result from extracellular fluid volume. For this reason, diuretics have been the cornerstone of heart failure treatment for more than 200 years. During the past 20 years, approaches to treatment have changed dramatically. Angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (AT1 blockers), beta-blockers and low dose spironolactone have become the drugs of choice to treat patients with systolic dysfunction (Ellison, 2001). Despite the dramatic improvement in mortality rates engendered by these therapeutic approaches, loop (and DCT) diuretic remain essential component of treatment regimens. The clinical efficacy of ACE inhibitors (and probably AT1 receptor blockade) improving mortality rates and their action on kidney suggested to some investigator that these drugs might obviate the need for diuretics. Several studies have indicated, however that they do not. It is found that diuretic requirement did not decline when patients with moderate or severe congestive heart failure were begun on ACE inhibitor. In fact, when the mean diuretic dose was reduced by 50% at the time ACE inhibitor were initiated, congestive symptoms resumed in almost every case. The explanation for the failure of ACE inhibitor to reduce ECF volume lies in their dual effect. Diuretic drugs shift the renal function curve to the left, permitting sodium excretion to increase. ACE inhibitors also shift the renal function curve to left, but additionally reduce mean arterial pressure because they are potent vasodilators (Odumujiccoa et al., 1989). The net effect of ACE inhibitors in most patients is to produce little
change in urinary sodium secretion because of the shift in the renal function curve and the reduced blood pressure offset.

The mechanism for diuretic resistance in patients with congestive heart failure appears to involve both pharmacokinetic and pharmacodynamic causes. In turn, the pharmacokinetic change is dependent on patient's level of renal function (Brater et al., 1984). Most patients with congestive heart failure, however, have some degree of renal impairment if not intrinsic then secondary to heart disease. As a consequence, most will require doses two fold to three fold higher than normal in order to attain normal amount of diuretic in the urine. In turn such doses represent ceiling doses in this clinical condition. As far as the pharmacodynamic components are concerned, the proximal and distal tubular reabsorption of sodium are stimulated in severe heart failure. This is due to (i) direct proximal tubular effects of angiotensin II and the resultant facilitation of passive sodium reabsorption in the proximal tubule and (ii) catecholamine mediated efferent arteriolar constriction with subsequent high peritubular capillary oncotic pressure and aldosterone mediated sodium reabsorption in the principal cells of collecting ducts (Anand et al., 1989; Anand et al., 1989a; Fliser et al., 1997). In addition, resistance to atrial natriuretic peptide, despite high plasma levels, may contribute to sodium retention (Cavero et al., 1990). Beneficial effect of an ACE inhibitor suggest that renin-angiotensin system is involved in sodium retention (Volpe et al., 1997) see figure 2.

5.2 Chronic renal insufficiency

A loop diuretic is the diuretic of choice in patients with renal insufficiency. Although a large dose of a thiazide will cause diuresis in patients with mild renal insufficiency, the response in patients with a creatinine clearance of less than about 50 ml per minute is poor.

Predictably, decreased renal function results in a concomitant decrease in the renal clearance of loop diuretic and the resultant decreased delivery to the urinary site of action. The degree to which renal
clearance is decreased differs among the loop diuretics. In subjects with normal renal function, approximately 50% of an intravenous dose of furosemide reaches the urine (Voelker et al., 1987), whereas in patients with renal insufficiency only about 10% of an intravenous dose reaches the urine. However, only 5% of bumetanide dose reach the urine in patient. Torasemide follows a pattern similar to that of bumetanide. The reason for the difference between bumetanide and torasemide on the one hand contrasted with furosemide on the other hand is that former two diuretic follow different route of non-renal elimination from furosemide. Bumetanide and torasemide are metabolised, presumably via cytochrome P450 pathways in the liver that are unaffected by renal disease. These non-renal elimination pathways largely remain intact in patients with severe renal insufficiency. In contrast, the non-renal elimination of furosemide occurs via glucoronidation, a substantial component of which may occur in kidney (Brater, 1989). This non-renal elimination of furosemide is diminished in patients with severe renal insufficiency, but mechanism is unknown. The net result of this effect is that elimination of furosemide by both renal and non renal routes is compromised in patients with severe renal disease, causing persistently high serum concentration of furosemide which in turn provide more diuretic to be secreted into urine despite the severely depressed renal clearance. The clinical corollary of this phenomena that less dose escalation is needed with furosemide in order to deliver 'normal' amount of diuretic into urine; thus only five fold higher doses than normal are needed in such patients where as bumetanide and torasemide require ten fold higher doses. Even if a fractional excretion of sodium is attained in patient with severe renal insufficiency, however the amount of total sodium excreted is still small because filtered sodium becomes limiting factor. Therapeutic strategy for such patient is dose calculation on the basis of renal insufficiency or constant intravenous infusion (Rudy et al., 1991).
A frequent question is, what is the largest single dose of a loop diuretic that can be given to a patient with severe renal insufficiency? The maximal natriuretic response occurs with intravenous bolus doses of 160 to 200 mg of furosemide or the equivalent doses of bumetanide and torsemide (Voelkar et al., 1987; Rudy et al., 1994), and nothing is gained by using larger doses. Some patients may require these large doses several times a day. Single intravenous bolus doses of 160 to 200 mg can occasionally cause transient tinnitus, but administering the dose over a period of 20 to 30 minutes can minimize this effect. In patients who have poor responses to intermittent doses of a loop diuretic, a continuous intravenous infusion can be tried. Before administering a continuous infusion of loop diuretic, the physician should give a loading dose in order to decrease the time needed to achieve therapeutic drug concentration. The rate of the continuous infusion is governed by the patient’s renal function. Another strategy to enhance the response to a loop diuretic is to add an oral thiazide diuretic. In summary, a patient with edema caused by renal insufficiency should be given increasing doses of a loop diuretic until an effective dose is identified. The effective dose should be given as often as needed to maintain the response, according to the patient's ability to restrict sodium intake and the duration of action of the drug. If the response is inadequate after the maximal dose has been reached, a thiazide should be added. If diuresis remains inadequate, the only recourse is dialysis.

5.3 Nephrotic syndrome

Patients with nephrotic syndrome, who even have normal glomerular filtration rates, deliver adequate amounts of diuretic into the urine, but they still do not respond as briskly as do healthy subjects. Even though normal total amounts of diuretics are delivered into the urine, studies in experimental animals indicate that the albumin present in the luminal contents as a result of the disease process is capable of
binding the diuretic and thereby preventing its access to the Na-K-2Cl reabsorptive pump (Krichner et al., 1990).

The degree to which binding occurs cannot be measured in human subjects but extrapolation from *in vitro* and animal data would suggest that one half to two thirds of all diuretics appearing in the urine of a nephrotic subject could be bound and thereby inactivated by protein urea. As such, doses twofold to threefold higher than normal would be needed to provide normal amounts of unbound furosemide to the site of action. These data allow prediction of the amount of intravenous loop diuretic that would constitute a ceiling dose in patients with nephrotic syndrome. It should be emphasized that these estimates apply only to patients with normal glomerular filtration rates. If glomerular filtration is also compromised, the ceiling dose must be concomitantly increased to compensate for the decrease in glomerular filtration rate.

It has also been speculated that in patients with nephrotic syndrome there is an increase in the volume distribution, so that serum concentration of loop diuretic are sufficiently low to limit delivery of diuretic into the urine. Exploration of this phenomenon has resulted in the speculation that administration of the combination of albumin and furosemide to patients with nephrotic syndrome might elicit response in patients who do not otherwise respond (Brater, 1994). Several studies, however, documented that patients with nephrotic syndrome deliver normal amounts of diuretic into urine (Krichner et al., 1990). Thus, a therapeutic strategy of administration of albumin plus loop diuretic seems irrational in the majority of patients with nephrotic syndrome.

5.4 Hepatic cirrhosis

The mainstay of diuretic therapy for patients with cirrhosis who have edema is spironolactone, because secondary hyperaldosteronism is an important cause of sodium and water retention in such patients. Spironolactone causes only a moderate diuresis, which is desirable
because greater diuresis may compromise the intravascular volume (Sheer et al., 1970).

In hepatic cirrhosis, bumetanide and torasemide has increased delivery of diuretics into the urine compared with healthy subjects, because non renal clearance of these diuretics is diminished concomitant with decreased hepatic function. It is clear however, that patient with cirrhosis do not respond adequately and so the mechanism of diuretic resistance must be pharmacodynamic (See Fig. LR 2).
Fig. LR 2: Algorithm for diuretic therapy in patients with edema caused by renal, hepatic, or cardiac disease.
6.0 TREATMENT OF DIURETIC RESISTANCE

Before considering intensive diuretic therapy or combination therapy for the treatment of diuretic resistance, it is necessary to exclude reversible causes of diuretic resistance. An inadequate reduction in ECF volume does not necessarily indicate an inadequate natriuretic response, as loop diuretics may induce natriuresis without contracting the ECF volume if dietary NaCl intake is excessive. It should also be emphasized that the desired ECF volume may not be associated with an edema free state and consideration should always be given to the need to reduce ECF volume further. Understanding mechanisms of diuretic action and adaptation, the causes of diuretic resistance and goals of diuretic treatment helps one to select an optimal approach to the diuretic resistant patient.

6.1 High dose diuretic therapy

High doses of loop diuretics are frequently employed to treat severe volume overload, especially when treatment is urgent. Maximal effective doses of furosemide, bumetanide and furosemide have been estimated. When given as bolus maximal effective doses of furosemide range from 50 mg in hepatic cirrhosis to 500 mg IV in severe acute renal failure. Despite these estimates, some investigator have used much higher doses of furosemide and reported therapeutic success (Gerlag and Van Meijel, 1988). Hypokalemia, hyponatremia and hypotension frequently result because of excessive fluid and electrolyte losses. For diuretic resistant patients, however, drug toxicity, most commonly ototoxicity, may also occur and is an important consideration during high dose or prolonged therapy (Ryback, 1993). It is generally reversible but occasionally irreversible. It appears to be related to serum concentration of the drug. It has been suggested, and the clinical experience seems to confirm, that ototoxicity of furosemide can be minimized by administering it no faster than 15 mg/min (Ellison, 1999). Comparable data are not available for bumetanide and torasemide but it seems reasonable to avoid rapid
bolus. Myalgias appear to be more common following high doses of bumetanide. The avoidance of high peak levels and concomitant toxicity is one reason that continuous infusion of diuretics has become popular as an alternative approach to treat diuretic-resistant patients.

Although venodilation and improvements in cardiac hemodynamics frequently result from intravenous therapy with loop diuretics, more recent reports suggest that the hemodynamic response to intravenous loop diuretic may be more complex. It is reported that low dose furosemide increased venous capacitance via prostaglandin mediated vasodilatory action but higher doses did not (Johnson et al., 1984). It was suggested that furosemide induced renin secretion led to increase in angiotensin II generation which overwhelms vasodilatation. In two series 1 to 1.5 mg/kg furosemide boluses, administered to patients with chronic congestive heart failure, resulted in transient deterioration in hemodynamics during first hour (Francis et al., 1985; Curran et al., 1992). In patients who are already treated with an angiotensin I-converting enzyme inhibitor, immediate symptomatic improvement is likely to result from diuretic administration. Another complication of high dose furosemide treatment may be thiamine deficiency. In humans, some but not all workers have detected thiamine deficiency in patients treated chronically with furosemide. In one study, patients with congestive heart failure who received furosemide 50 mg daily for at least 3 months were randomized to receive intravenous thiamine or placebo. Intravenous thiamine led to improved hemodynamics and natriuresis, compared with placebo and to an improvement in the thiamine-pyrophosphate effect on erythrocyte transketolase activity (Shimon et al., 1995).

6.2 Combination diuretic therapy

A common and useful method for treating the diuretic-resistant patient is to administer 2 classes of diuretic drugs simultaneously. Some authors have advocated alternating 2 members of the same diuretic class together; controlled trials suggest little or no benefit from such an
approach (Chemtob et al., 1989). In contrast adding a proximal tubule diuretic or distal convoluted tubule diuretic to a regimen of loop diuretic is often dramatically effective (Epstein et al., 1977; Oster et al., 1983; Oimomi et al., 1990).

DCT diuretics are the class of drugs most commonly added to loop diuretics and this combination has proved to be remarkably effective. The combination of loop and DCT diuretic has been shown to be synergistic in formal permutation trials (Ellison, 1991). The addition of DCT diuretics to loop diuretics may enhance sodium chloride excretion by several mechanisms, none of which is mutually exclusive. The first mechanism responsible for the efficacy of combination therapy is that DCT diuretics may prevent or attenuate post diuretic NaCl retention. The natriuretic effects of a single dose of furosemide, bumetanide and to a lesser extent torasemide, generally cease within 6 hours. Before the next dose of diuretic is administered, intense renal NaCl retention frequently occurs (post diuretic NaCl retention); this NaCl retention can be attenuated by DCT diuretics which will continue to inhibit renal NaCl reabsorption after the loop diuretics has worn off.

A second mechanism by which DCT diuretic potentiates the effects of loop diuretic is by inhibiting salt transport along the proximal tubule. When the kidney is strongly stimulated to retain NaCl, proximal NaCl reabsorption is enhanced. Most thiazide diuretic inhibits carbonic anhydrase, thereby reducing Na and fluid reabsorption along the proximal tubule. This leads to increased Na and fluid delivery to the loop of Henle, which lead to increase in delivery of Na and fluid into collecting duct system. Hence, the delivery of solute to distal nephron will be greatly by magnified (Okusa et al., 1989).

A third mechanism is that the chronic loop diuretic administration leads to hypertrophy and hyperplasia of distal cells. Because DCT diuretics can inhibit thiazide sensitive NaCl cotransport completely even under these stimulated conditions, the effects of thiazide will be greatly
magnified in the patients who have developed distal nephron hypertrophy from high doses of loop diuretics (Ellison, 1987). Evidence of an effect of chronic loop diuretic treatment to enhance distal nephron function in human was obtained (Loon et al., 1989). They showed that the effect of chlorothiazide on urinary Na excretion was enhanced following one-month treatment with furosemide.

The most common approach for combination diuretic therapy is to add a DCT diuretic to a regimen of a loop diuretic. The dose of loop diuretic should not be altered. The shape of the steep dose response curve of loop diuretic is not affected by the addition of other diuretics and the loop diuretic must be given in an effective or maximal safe dose. The choice of DCT diuretic to add is arbitrary. Many clinicians choose metolazone because its half-life is longer than that of some other DCT diuretics and has been reported to remain effective even when the glomerular filtration rate is low (Ellison, 1999). However, direct comparisons between metolazone and several traditional thiazides have shown little difference in natriuretic potency when included in a regimen with loop diuretics in patients with nephrotic edema, congestive heart failure, and azotemia.

DCT diuretics may be added in full doses when rapid and robust response is required but such an approach is likely to lead to complications, if intense follow up is not done. It may be best reserved for hospitalized patients because fluid and electrolyte depletion, sometimes massive, occurs commonly. To prevent or minimize the side effects, there are two approaches:

- Achieve control of extracellular fluid volume by adding full doses of DCT diuretics on daily basis initially and then to maintain control by reducing the administration of DCT diuretic to 3 times weekly. The other advantage of this is that it prevents DCT diuretic induced down regulation of Na/K ATPase activity (Garg and Narang, 1987) and transport activity along distal convoluted tubule of rat.
Another approach to combination therapy is to use combination therapy for only a fixed course. A comparison of different combination diuretic regimens suggested that a limited course of combination therapy might be as effective and perhaps safer than more prolonged courses (Channer et al., 1994). Because DCT diuretics are absorbed more slowly than loop diuretics, it may be reasonable to administer DCT diuretic 1/2 to 1 hour prior to loop diuretic.

Drugs that act along the collecting duct i.e. amiloride and spironolactone can be added to a regimen of loop diuretic drugs, but their effects are generally less dramatic than those of DCT diuretics. The combination of spironolactone and loop diuretic has not been shown to be synergistic, but these drugs have important roles in preventing hypokalemia while maintaining renal Na excretion. The setting in which collecting duct diuretic are used most commonly is to treat patients with cirrhosis of the liver in whom hypokalemia must be avoided because it can predispose to hepatic encephalopathy (Brater, 1998).

Recently a role of spironolactone in treating patients with congestive heart failure has been suggested. This follows from the observations:

- Patients with congestive heart failure who are being treated with angiotensin I converting enzyme inhibitors (ACEI) frequently 'break through' manifesting elevated levels of aldosterone despite doses of ACEI (Zannad, 1993).
- Mineralocorticoid receptors are located throughout the body, including on cardiac tissue. Recent data suggests that hyperaldosteronism may contribute to myocardial dysfunction directly (Weber and Villarreal, 1993).
- Arrhythmias associated with hypokalemia and hypomagnesia.
- Hypotension associated with ACEI
One situation in which aggressive diuretic therapy is often indicated is for hospitalised patients. Two intravenous drugs are available to supplement loop diuretic for combination therapy. Chlorothiazide (500-1000 mg one or twice daily) and acetazolamide (250-375 mg up to four times daily) are both available for intravenous administration. In many situations, combination diuretic therapy may be targeted at underlying disease process. Theophylline is a very mild diuretic, but it has been shown to act synergistically with loop diuretics and may be useful when bronchospasm and edema are present together (Brater et al., 1983).

6.3 Continuous diuretic infusion

For hospitalized patients who are resistant to diuretic therapy, another approach is to infuse diuretic continuously. It has several advantages:
1. They avoid troughs of diuretic concentration, thereby preventing intermittent periods of positive NaCl retention. A constant infusion leads to constant urinary concentration.
2. Constant infusions appear to be more efficient than bolus therapy. In one study of patients with chronic renal failure, the continuous infusion of bumetanide was 32% more efficient than bolus of same dose when the amount of NaCl excreted per mg of administered was compared (Rudy et al., 1991).
3. Some patients who are resistant to large doses of diuretics given by bolus have responded to continuous infusion (Gerlag and Van Meijel, 1988).
4. Drug toxicity from loop diuretics, such as ototoxicity and myopathies appear to be less common when drugs are administered as continuous infusion.

6.4 Ultra-filtration

When medical diuretics fail despite addressing consideration, plasma ultra-filtration may be considered. Ultra-filtration with or without
accompanying hemodialysis effectively removes extracellular fluid. Many clinicians have observed surprising beneficial effects of ultra-filtration in diuretic resistant patients. Agostoni et al. (1994) randomized patients with congestive heart failure to volume removal by ultra-filtration or furosemide. Regimens were devised to remove equal amounts of fluid. Whereas both approaches achieved same volume depletion, volume contraction was maintained significantly better following ultra-filtration than following medical diuretics. Thus, ultra-filtration may have a role in the rare patients with extracellular fluid volume overload who cannot be controlled using one of the medical approaches.

So frequently, combination of diuretic therapy is given to patients who are refractory to large doses of loop diuretic. Several studies have documented synergistic nature of these diuretic combinations in setting of severe disease. Hence, the present study was planned to evaluate the effect of combination diuretic therapy hydrochlorothiazide (50 mg) plus furosemide (40 mg) in comparison to furosemide (40 mg), furosemide (50 mg), hydrochlorothiazide (50 mg), spironolactone (50 mg) and placebo on electrolytes, urine volume, body weight and arterial blood pressure in healthy human subject.

7.0 STUDY DRUGS
7.1 FUROSEMIDE
7.1.1 Pharmacology

Furosemide is a potent diuretic acting primarily on the medullary portion of ascending limb of loop of Henle to inhibit to Na⁺-K⁺-2Cl⁻ cotransporter, which normally mediates ionic reabsorption. Furosemide inhibits sodium and potassium reabsorption by competing for the chloride binding site on the co-transporter at the luminal face of the epithelial cells. Furosemide is a weak inhibitor of carbonic anhydrase activity but the effect on the proximal tubule is negligible unless massive doses are used. Furosemide causes an increased loss of calcium and
magnesium, as well as of sodium potassium and chloride in the urine (Reeves and Molony, 1985; Wilson et al., 1983).

### 7.1.2 Clinical Pharmacology

In humans' intravenous, an intramuscular and oral dose of furosemide increases the urinary excretion of sodium (Na), chloride (Cl) and water. Urinary excretion of calcium and magnesium are also increased, although the loss of calcium may decrease during long-term treatment. It also increases the excretion of bicarbonate in the urine and the urinary pH rises. It causes a decrease in the renal secretion of uric acid & thus urate levels in the blood may rise causing gout. Furosemide has a wide dose-response curve, although in non-edematous subjects the increase is not linear.

Oral doses in the range 20 to 80 mg produce a linear increase in excretion of sodium, potassium and water in normal subjects. The diuresis is complete within 6 h. intravenous bolus doses can produce urine flow rates which are a substantial fraction of glomerular filtration rate (GFR). Intravenous injections of furosemide cause a transient increase in renal plasma flow in normal subjects, accompanied by an increase in GFR, which is also transient (Passmore et al., 1989).

There has been much discussion of tolerance to loop diuretics such as furosemide. Two mechanisms can be distinguished: homeostatic responses and disease progression. Use of loop diuretics, particularly in high doses, causes volume contraction and reduction in renal perfusion. Plasma renin concentrations rise and secondary aldosteronism occurs. Furosemide causes hypertrophy in tubular segments beyond the loop of Henle by increasing the delivery of luminal fluid to these segments. Locally increased sodium reabsorption is associated with this hypertrophy and this probably contributes to the development of tolerance to the natriuretic effects of furosemide.
7.1.3 Indications:
1. Acute Pulmonary edema
2. Chronic Congestive Cardiac failure
3. Hypertension
4. Edema caused by renal failure of nephrosis
5. Edema caused by liver cirrhosis
6. Hypercalcemia
7. Forced diuresis in treatment of poisoning

7.1.4 Absorption, Distribution, Metabolism, and Excretion

When oral doses of furosemide are given to normal subject, the mean bioavailability of the drug is approximately 52% but the range is wide, 27-80%. Food intake reduces the bioavailability of furosemide by about 80%. In plasma, furosemide is extensively bound to plasma protein, mainly to albumin. The volume of distribution of furosemide ranges from 170-270 ml/kg⁻¹. Presystemic metabolism is nil. Renal excretions of unchanged drug and elimination by metabolism plus fecal excretion contribute almost equally to the total plasma clearance.

Furosemide is in part cleared via the kidneys in the form of the glucuronide conjugate. A small amount (2% of the dose) enters the gut by passive diffusion from the plasma. The main pathway of non-renal clearance has not been identified but it does not appear to be hepatic and is partially probenecid-sensitive. Placental transfer of furosemide has been shown in pregnant women with edema or hypertension. Furosemide passes into the breast milk.

7.1.5 Adverse Drug Reaction

Successful diuresis induced by furosemide is accompanied by potassium wastage, however extent tends to be less pronounced than in case of thiazide diuretic. The clinical symptoms of potassium deficit often are vague and may consist of not more than lethargy and muscle weakness. Serious potassium depletion may lead to potentially lethal cardiac arrhythmias particularly in patients receiving cardiac glycoside.
Other side effects are magnesium depletion which may lead to cardiac arrhythmias, carbohydrate intolerance, life threatening hyponatremia, ototoxicity which is irreversible at high doses. The risk of ototoxicity caused by furosemide is increased when drug is given concomitantly with aminoglycoside antibiotic.

High doses of furosemide may lead to serious sodium and fluid depletion and to a fall in intra-vascular volume. Postural hypotension and decrease of glomerular filtration rate may ensue.

7.1.6 Dosage

Oral treatment with furosemide is usually begun with 20-40 mg once to three times daily and doses are increased depending on the therapeutic response. In general, oral doses of 160-320 mg daily may be needed and should not be exceeded. However in patients with renal failure oral doses up to 1000 mg 1 day may be required (Dollery, 1999).

7.2 HYDROCHLOROTHIAZIDE

7.2.1 Pharmacology

Thiazide diuretic cause their main natriuretic effect by decreasing sodium and chloride reabsorption in the cortical segment of the thick ascending limb of the loop of Henle by inhibition of a specific Na⁺Cl⁻ co-transporter. A minor component of the diuretic effect of hydrochlorothiazide may be the result of carbonic anhydrase inhibition in the proximal tubule, resulting in decreased sodium & bicarbonate reabsorption. Most thiazides have carbonic anhydrase (CA)-inhibiting activity, which is usually expressed relative to hydrochlorothiazide: HCTZ has about 0.3% the activity of acetazolamide.

7.2.2 Clinical pharmacology

It increases bicarbonate, phosphate, magnesium excretion and sodium, chloride, potassium, calcium excretion is decreased. It may reduce GFR, as a result of volume contraction but this is usually of relevance only in patients with impaired renal function. The saluretic
dose-response curve for diuretic is modified by compensatory mechanism causing a fall in GFR and increase in aldosterone secretion with sodium depletion caused by higher doses.

Hydrochlorothiazide is effective in lowering blood pressure but the mechanism of its antihypertensive effect is unclear. It probably involves extracellular volume (ECV) contraction, reduced cardiac output and/or peripheral vascular resistance (vasodilation and pressor insensitivity). A recent development is the discovery that the chemically related compound diazoxide (also diabetogenic), a powerful vasodilator, is a potassium channel opener, a new class of potentially useful antihypertensive drugs.

7.2.3 Therapeutic Indication
1. Edema
2. Control of essential hypertension
3. Management of diabetes insipidus
4. Idiopathic hypercalciuresis and calcium nephrolithiasis
5. Osteoporosis
6. Exercise induced hyperkalemia

7.2.4 Absorption, Distribution, Metabolism and Excretion

Following an oral dose, absorption is 70% and mainly from duodenum and jejunum. Absorption efficiency is independent of dose. Though plasma levels and urinary excretion are, dose dependent, peak electrolyte excretion is independent of dose over the therapeutic range. Plasma protein binding is 40-64%. Hydrochlorothiazide has biphasic elimination profile, which is probably result of its slow release from tissues. The first (α) phase plasma half-life is 2.5 hours and second (β) phase is 8-12 hours. Hydrochlorothiazide is not metabolized in man and is excreted almost entirely (more than 95%) in urine. Onset of diuresis occurs with in 2 hours of administration, peaks at 3-4 hours & last 6-12
hours. Onset of antihypertensive effect is over 3-4 days and last up to 1 week after stopping therapy.

It accumulates in red cells by an unknown mechanism (possibly binding to CA) and readily crosses the human placenta.

Food and the volume of fluid ingested do not effect absorption (some conflicting reports absorption is increased by anticholinergic drugs).

7.2.5 Adverse Drug Reaction

Except severe hyponatremia (Syndrome of inappropriate antidiuretic hormone secretion) and idiosyncratic hypersensitivity reactions (steven johnson Syndrome, Systemic lupus erythematosus acute pancreatitis, acute allergic interstitial pneumonitis,) deaths were rarely directly attributed to hydrochlorothiazide. Hypokalemia is a relatively common finding in patients taking thiazide diuretic which may precipitate fatal cardiac arrhythmias in some patient having either preexisting hypertension or heart failure.

Volume depletion and electrolyte imbalance (hypokalemia, hypochloremic alkalosis, hyponatremia) are the common features of overdose. In children, an overdose of HCTZ may cause lethargy and coma (without electrolyte upset).

There is little evidence that long-term treatment of asymptomatic patients with thiazide diuretics for hypertension predisposes to the development of atherosclerosis, in spite of their effects on plasma lipoproteins.

Hyperglycaemia may present with thirst and recurrent urinary tract infections, although it is commonly mild and asymptomatic. Hyperuricemia is common and often asymptomatic but can present with an acute attack of gout or renal colic. A recognized, but often missed, symptomatic adverse effect of thiazide treatment is impotence (mechanism unknown), especially in diabetics. HCTZ may significantly reduce tear production by an unknown mechanism.
7.2.6 Dosage

For hypertension, the maximum recommended dose of hydrochlorothiazide in the UK is 50 mg daily. However, doses of up to 200mg daily have been used. For edema, the maximum recommended dose of hydrochlorothiazide in the UK is 75 mg daily. However, initial oral doses of 25-200 mg daily in 1-3 doses followed by 25-100 mg daily as maintenance have been used.

The doses as low as 6.2 mg twice a day may have a useful effect. A study which compared 3.6, 12.5 and 25 mg of hydrochlorothiazide, or placebo, in 111 patients with mild hypertension concluded that 12.5 mg hydrochlorothiazide had a borderline effect on blood pressure, while 25 mg had a definite antihypertensive effect (Dollery, 1999).

7.3 SPIRONOLACTONE

7.3.1 Clinical Pharmacology

Spironolactone is a competitive inhibitor of the binding of aldosterone to its receptor, the most important of which lie is late distal renal tubules and the renal collecting system. It combine with soluble cytoplasmic aldosterone receptor to form complexes which are inactive and which don't bind to nuclear acceptor site, thus preventing a chain of biochemical events leading to the synthesis of physiologically active protein. It is primarily useful as a diuretic in patients with hyperaldosteronism. It is effective in patients with ascites due to liver failure. Its anti hypertensive effects are relatively modest in essential hypertension but it is of value in the treatment of hypertension due to primary hyperaldosteronism where other definitive treatments are not feasible. And in patients with resistant heart failure (i.e. where other diuretics have failed). It is less useful as a first line diuretic.

7.3.2 Absorption, Distribution, Metabolism, and Excretion

Oral absorption of spironolactone is variable because of its low aqueous solubility. There is improved absorption if the drug is taken after food probably because of delaying gastric emptying, food promote
disintegration of tablet and improve dissolution of drug. The peak plasma concentration was observed at one hour (hr) in normal volunteers. After a standardized meal, systemic bioavailability has been 60-70% and plasma half-life is $1.3 \pm 0.3$ hr. Spironolactone is 98% protein bound but its volume of distribution is unknown. It is extensively metabolized to canrenone and other metabolites, which are also competitive antagonist of aldosterone. Furthermore, bile acids secreted in response to the meal may dissolve spironolactone, which is very lipophilic.

7.3.3 Adverse Drug Reaction

Drowsiness, Confusion, abdominal upset, gynaecomastia, impotence & menstrual irregularities

Most serious is hyperkalemia that may occurs especially in cirrhotics. Development of breast cancer has been reported in patients taking spironolactone chronically, but a causal relationship has never been established and it seems likely that the original observation was a chance event.

Spironolactone may cause hyperkalemia and hyponatremia and deterioration in renal function may occur, particularly in those with pre-existing renal impairment.

Hyperchloremic metabolic acidosis has been reported in advanced hepatic cirrhosis. Gastrointestinal disturbances have been described including peptic ulceration and hematemesis.

7.3.4 Dosage

In secondary aldosteronism and essential hypertension, a daily dose of 50-100 mg may be sufficient, where as in the diagnosis and treatment of primary hyperaldosteronism. It may be necessary to prescribe up to 100 mg daily (Dollery, 1999b).
MATERIALS,
METHODS & SUBJECTS
1. OBJECTIVE OF THE STUDY

1. The primary objective of the study was to evaluate in healthy subject, the multiple dose effects of furosemide (40mg) plus hydrochlorothiazide (50mg) and furosemide (40mg) plus spironolactone (50mg) combinations in comparison to furosemide (40mg), furosemide (80mg) hydrochlorothiazide (50mg), spironolactone (50 mg) and placebo on the electrolytes and urinary volume.

2. The secondary objective of the study was to evaluate the effect of above-mentioned interventions on the body weight and arterial blood pressure if any.

A pilot study was conducted with furosemide (40mg) to validate the phenomena of development of resistance by evaluating its effect on urinary electrolytes and urine volume.

2.0 PILOT STUDY

2.1 Study Design

A randomised, single blind, two treatment, two period, crossover pharmacodynamic study to evaluate the effects of multiple dose furosemide (40 mg) and placebo on the urinary electrolytes and urinary volume, in healthy, adult, male, human subjects under fasting conditions.

3.0 STUDY MEDICATION AND RANDOMISATION

Furosemide (40mg)

Tablets containing 40mg furosemide, manufactured by Aventis Pharma, India.

Placebo

Capsules containing lactose manufactured by Department of Pharmaceutics Faculty of Pharmacy, Hamdard University, and India.

After the preparation, formulations were stored under prescribed storage condition in a controlled access area.
The order of receiving the treatment for each subject during the 2 periods was determined according to blocked random number table.

**RANDOMISATION CODE (n=6)**

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A = Furosemide 40mg, B = Placebo Caps

**4.0 NUMBER OF SUBJECTS**

Adequate number of healthy adult male human subject were enrolled to allow dosing of 6 subject in the first period.

**4.1 SELECTION OF SUBJECTS**

**4.1.1 Inclusion Criteria**

1. Be in the age range of 18-45 years.
2. Be neither underweight nor overweight for his height as per the stipulation of life insurance Corporation of India Height/weight chart for non-medical cases.
3. Have given written informed consent to participate in this study.
4. Be of normal health as per determined by medical history and examination of the subjects performed within 15 days prior to the commencement of the study.
5. The following lab determinations were performed.
   i. Presence or absence of disease markers of HIV, Hepatitis B virus and syphilitic infections.
   ii. Levels of hemoglobin, total leukocyte count, differential count and erythrocyte sedimentation rate
iii. Values of serum creatinine, serum aspartate amino transferase, serum alkaline phosphatase, serum bilirubin, fasting plasma glucose and serum cholesterol.

iv. Chemical and microscopic examination of urine.

4.1.2 Exclusion Criteria
1. History of allergy to furosemide or lactose.
2. Any evidence of organ dysfunction or any clinically significant deviations from the normal in physical and clinical determinations.
3. Physical handicap or disability.
4. History of serious head injury or neurological disorder.
5. History of psychiatric illness, which may impair the ability to provide, written informed consent.
6. Regular smoker more than 20 cigarettes per day and have difficulty in abstaining from smoking during the study period.
7. Use of enzyme modifying drug within 30 days of any systemic medication (including OTC) within 14 days prior to the day 1 of the study.
8. Participation in any clinical trial within 6 weeks preceding day of the study.

5.0 ADMISSION
Subjects were asked to maintain their diet for duration of study and in particular adhere to same pattern of meal in the 48 hour preceding to the first dose.

In each periods, subjects were admitted to the CPU in the evening. They were dosed after the overnight fasting. Urine collection was by spontaneous voiding and done at predetermined intervals.

6.0 DOSING
Dose administration as described below was done under supervision of a trained study personnel.

a) A single oral dose of a placebo capsule was administered with 240 mL of potable water at ambient temperature after an overnight fast of 10-
b) A single oral dose of a furosemide (40mg) tablet was administered with 240 mL of potable water at ambient temperature after an overnight fast of 10-12 hours.

7.0 EVALUATION PARAMETERS
The following measurements were done for evaluation of the study drugs
1. Urine Volume
2. Urinary concentration of sodium
3. Urinary concentration of potassium
Urine collection was by spontaneous voiding on Day 1, Day 3, and Day 5 of drug administration
Collection of urine was done at 0 to 30, 30 to 60, 60 to 90, and 90 to 120 minutes and at 2 to 4, 4 to 6 hour (hr) after drug administration.
Fluid replacements: Fluid replacement was given by providing equivalent volume of drinking water to each volunteer after every urination, to make up for the water loss.

8.0 RESTRICTIONS
8.1 Medications
Subjects should not have received any medication during the two weeks period prior to the start of the study. They were instructed during screening not to take any prescription and OTC medications subsequently until the completion of the study. If necessary, they were asked to consult the investigator.

8.2 Diet
All the subjects were instructed to abstain from any xanthine containing food, beverages, or alcoholic products for 48 hours prior to dosing and during housing in each period. In the study, they were asked to fast over night (10 – 12 hours) before dosing and four hours post dose. A standard
lunch and snacks were provided after 4 hr and 6 hr of the dosing.

9.3 Activity
All subjects were dosed while seated and instructed to remain seated or ambulatory for the first two hours following each drug administration. Thereafter, subjects were allowed to engage only in normal activities while avoiding severe physical exertion.

9.0 HANDLING OF SAFETY PARAMETERS

9.1 Clinical Safety Measurements
Vital signs of oral temperature, sitting blood pressure and radial pulse were measured during subject check-in, prior to each dosing and two hours, and before checkout in each period. Vital signs to be measured prior to administration of the dose were taken within one hour of the scheduled dosing time. At all other times, vital signs were taken within 30 minutes of the scheduled times.

Brief clinical examination of the subjects was conducted by a qualified medical designate on duty after subject check-in, prior to dosing of study drug and before checkout.

9.2 Adverse Events
Subjects were monitored throughout the study period for adverse events. Subjects were informed to bring to the notice of the nurse or the doctor any adverse event that may occur during their stay at the site of investigation.

10.0 ETHICAL CONSIDERATIONS

10.1 Basic Principles
This research was carried out in accordance with the Clinical Research guidelines defined in the U.S. 21 CFR part 312.20 and the principles enunciated in the Declaration of Helsinki (South Africa 1996).

10.2 Institutional Review Board
This protocol and the corresponding informed consent form (ICF) used to obtain informed consent of study subjects was reviewed by the Jamia Hamdard Institutional Review Board.
10.3 Informed Consent
Subjects were informed before the initiation of study through an oral presentation regarding the purpose, procedures to be carried out, potential hazards and rights of the subjects. Subjects were required to understand and sign a consent form summarizing the discussion prior to check-in for the study in Period I. They were also informed about their right to leave and to ask any question at any time related to the project. A copy of signed ICF was given to them.

10.4 Volunteer Compensation
The subjects were adequately compensated on account of their participation in the study. In case of drop-out/withdrawal of a subject before completion of the study, the guidelines issued by the Jamia Hamdard Institutional Review Board were final.

11.0 STATISTICAL ANALYSIS
Statistical analysis was performed on the data from subjects. If necessary, an unequal number of subjects per sequence were used. Paired t test and analysis of variance test were applied. A difference was considered significant when the probability of type I error is less than 5% (p<0.05)

12.0 DEFINITIVE STUDY
12.1 Study Design
A randomised, single blind, crossover, placebo controlled, seven treatment, seven period, multiple dose pharmacodynamic study to evaluate the effect of furosemide (40 mg) plus hydrochlorothiazide (50 mg) and furosemide (40 mg) plus spironolactone (50 mg) combination in comparison to furosemide (40 mg) furosemide (80 mg), hydrochlorothiazide (50 mg), spironolactone (50 mg) & placebo on the urinary electrolytes, urinary volume, blood electrolytes, body weight & arterial blood pressure if any.
13.0 STUDY MEDICATION

Furosemide (40mg)
Tablets containing 40mg furosemide, manufactured by Aventis Pharma, India.

Hydrochlorothiazide (50mg)
Two Tablets, each containing 25 mg hydrochlorothiazide manufactured by Sun Pharma, India.

Spironolactone (50mg)
Two tablets, each containing 25mg spironolactone, manufactured by RPG Life Sciences, India.

Placebo
Capsules containing lactose manufactured by Department of Pharmaceutics Faculty of Pharmacy, Hamdard University, India.

14.0 RANDOMIZATION
The randomization schedule was generated using the SAS statistical software.
### Randomization Code (N=14)

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</table>

A = Furosemide 40mg, B = Furosemide 80mg, C = hydrochlorothiazide 50mg, D = Spironolactone 50mg, E = Hydrochlorothiazide 50mg + furosemide 40mg, F = Spironolactone 50mg + Furosemide 40mg, G = Placebo caps.

### 15.0 Blinding Procedure

Subjects were unaware as to which drugs they were receiving. The blinding was done by providing medicament in the capsules.

### 16.0 Number of Subjects

Adequate number of healthy adult male human subjects were enrolled to allow dosing of 15 subjects in the first period. Subsequent drop-out were
not replaced. If necessary, an unequal number of subject per sequence was used.

16.1 Inclusion Criteria
1. Be in the age range of 18-45 years.
2. Be neither underweight nor overweight for his height as per the stipulation of life insurance Corporation of India Height/weight chart for non-medical cases.
3. Have given written informed consent to participate in this study.
4. Be of normal health as per determined by medical history and examination of the subjects performed within 15 days prior to the commencement of the study.
5. The following lab determinations were performed.
   v. Presence or absence of disease markers of HIV, Hepatitis B virus and syphilitic infections.
   vi. Levels of hemoglobin, total leukocyte count, differential count and erythrocyte sedimentation rate
   vii. Values of serum creatinine, serum aspartate amino transferase, serum alkaline phosphatase, serum bilirubin, fasting plasma glucose and serum cholesterol.
   viii. Chemical and microscopic examination of urine.

16.2 Exclusion Criteria
1. History of allergy to furosemide, hydrochlorothiazide, spironolactone and lactose
2. Any evidence of organ dysfunction or any clinically significant deviations from the normal in physical and clinical determinations.
3. Physical handicap or disability.
4. History of serious head injury or neurological disorder.
5. History of psychiatric illness, which may impair the ability to provide, written informed consent.
6. Regular smoker more than 20 cigarettes per day and have difficulty in abstaining from smoking during the study period.

7. Use of enzyme modifying drug within 30 days of any systemic medication (including OTC) within 14 days prior to the day 1 of the study.

8. Participation in any clinical trial within 6 weeks preceding day of the study.

17.0 ADMISSION

Subjects were asked to maintain their diet for duration of study and in particular adhere to same pattern of meal in the 48 hour preceding to the first dose.

In each period, subjects were admitted to the CPU 24 hours prior to dosing. They were provided with standard breakfast, lunch and dinner. Also they were given fixed volume of water (1.5 litres) load during their in house stay prior to dosing. This was done to ensure homogenous salt and water load among the volunteers. Twenty four hour urine collection was made preceding the first dose. This was done with the intention to measure daily sodium and water excretion and hence to ensure maintenance of the sodium and water balance during study period.

They were dosed after the overnight fasting. Urine collection was by spontaneous voiding and done at predetermined intervals.

18.0 DRUG ADMINISTRATION

Dose administration as described below was done under supervision of a trained study personnel.

Treatment A

A single oral dose of a furosemide (40mg) tablet was administered with 240 mL of potable water at ambient temperature after an overnight fast of 10-12 hours.

Treatment B

A single oral dose of a furosemide (80mg) tablet was administered with
240 mL of potable water at ambient temperature after an overnight fast of 10-12 hours.

**Treatment C**
A single oral dose of a hydrochlorothiazide (50mg) tablet was administered with 240 mL of potable water at ambient temperature after an overnight fast of 10-12 hours.

**Treatment D**
A single oral dose of two spironolactone (25mg) tablets was administered with 240 mL of potable water at ambient temperature after an overnight fast of 10-12 hours.

**Treatment E**
A single oral dose of a furosemide (40mg) tablet + hydrochlorothiazide (50mg) tablet was administered with 240 mL of potable water at ambient temperature after an overnight fast of 10-12 hours.

**Treatment F**
A single oral dose of a furosemide (40mg) tablet + two spironolactone (25mg) tablets was administered with 240 mL of potable water at ambient temperature after an overnight fast of 10-12 hours.

**Treatment G**
A single oral dose of a placebo capsule was administered with 240 mL of potable water at ambient temperature after an overnight fast of 10-12 hours.

**19.0 EVALUATION PARAMETERS**
The following measurements were done for evaluation of the study drugs
1. Urine Volume
2. Urinary concentration of sodium
3. Urinary concentration of potassium
4. Serum concentration of sodium
5. Serum concentration of potassium
6. Body weight measurement
7. Arterial blood pressure
Urinary sodium and potassium were estimated by systronic Flame photometer.

19.1 Flame Photometry
Systronics flame photometer "Mediflame" 127 is a dual channel instrument, capable of quick and simultaneous estimation of sodium (Na) and potassium (K). It is especially designed for clinical analysis. The respective channels digitally display-estimated results. This minimizes the operation time, sample consumption and cost of operation.

The principle of operation is simple. The fluid under analysis is sprayed as a fine mist into the non-luminous flame, which becomes colored according to the characteristic emission of the element. The flame is simultaneously monitored by both the channels. Each channel consists of detector which views the flame through a narrow band optical filter that only passes the wavelengths centered around a specific emission of the selected elements (Na: 589 nm, K: 768 nm). The output of the detector is connected to an electronic metering unit, which provides digital readouts. Before analyzing the unknown fluids, the system is standardized with solutions of known concentration of elements of interest.

Operating Procedure and Sample Estimation
Once the burner was ignited and set, the steps were followed as described below:

1) Mains supply to the unit was switched on.
2) The 'Set F. S. Coarse' and 'Fine Controls' were turned in the maximum clockwise position.
3) Appropriate filter was selected with the help of the filter selector wheel.
4) Distilled water was fed to the atomizer for at least 30 seconds.
5) The 'set ref. coarse' and 'fine controls' were set to display readout of zero for K only.
6) 1 mEq per liter of Na solution (or the standard 1.0/0.01 mEq per litre of Na/K solution) was aspirated. After 30 seconds of aspiration, the
'SET REF COARSE' and 'FINE CONTROLS' were adjusted to give out a reading of 100 on the Na display.

7) 1.7/0.08 mEq per liter of Na/K solution was aspirated. After 30 seconds of aspiration, the 'Set F. S. control' of Na-side was adjusted to give the readout of 170 and that of K-side for readout of 80. The unit was calibrated in this manner.

8) The steps 4, 5, 6, and 7 were repeated to calibrate the instrument. The unknown sample was then fed to the atomizer for at least 30 seconds to give out a display of the concentration of Na and K. Automatic electrolyte analyzer had performed measurements of serum sodium and Potassium. Three ml of blood sample was collected in sodium fluoride tube, centrifuged; serum was separated and stored under 2-8°C.

19.2 Basic Principle of Automatic electrolyte Analyzer

At equilibrium, an electrical potential is generated that is proportional to the logarithm of the analyte activity in the sample. The potential of the Sodium, Potassium, and the chloride electrodes is measured sequentially against the reference electrode by a high impedance electrometer. The reference electrode consists of a calomel electrode and Salt Bridge solution, which is recycled. The concentration of the desired ion is calculated from the difference in the electrode potential between the standard and the sample by using the Nernst Equation:

\[ C_{\text{sample}} = C_{\text{standard}} \times 10^{\Delta E/\text{slope}} \]

Where:
- \( C_{\text{standard}} \) = ion concentration in the sample
- \( C_{\text{sample}} \) = ion concentration in the standard
- \( \Delta E \) = difference (in millivolts) between the electrode potential of the sample and the standard.
- Slope = calibration slope in (millivolts/decade)
19.3 Blood Pressure measurement
Sitting Systolic, Diastolic Blood Pressures (SBP and DBP) respectively will be recorded by using mercury sphygmomanometer. Systolic blood pressure will be recorded as the value corresponding to the Korotkoff's Phase I and diastolic to Phase V.

20.0 EVALUATION SCHEDULES
Urine collection was by spontaneous voiding on Day 1 and Day 5 of drug administration
- For furosemide, collections were done at 0 to 30, 30 to 60, 60 to 90, and 90 to 120 minutes and at 2 to 4, 4 to 6, 6 to 8, and 8 to 12 hours (hr).
- For hydrochlorothiazide, spironolactone, and placebo, collections were done at 0 to 3, 3 to 6, 6 to 9 and 9 to 12 hr.
- For furosemide plus hydrochlorothiazide and furosemide plus spironolactone, collections were at 0 to 30, 30 to 60, 60 to 90, 90 to 120 minutes and at 2 to 3, 3 to 6, 6 to 9, 9 to 12 hr.

20.1 Fluid replacements
Fluid replacement was given by providing equivalent volume of drinking water to each volunteer after every urination, to make up for the water loss.

20.2 Clinical measurements
Blood sampling, Blood Pressure, and weight measurement was done at twelfth hour of each day of study

21.0 WASH OUT PERIOD
There was a washout period of at least six days between each study period.

22.0 RESTRICTIONS
Restrictions on medications, diet and activities in the CPU were same as described for the pilot study
23.0 ASSESSMENT OF COMPLIANCE

Compliance was assessed by conducting an on the spot thorough examination of the oral cavity by a trained study personnel after dosing during their stay in the unit. The subjects will have to take at least 80% of the tablets issued, in order to be considered as complaint with the protocol.

23.1 Counseling

Each Volunteer was specially counseled in each visit on daily consumption to have a maximum compliance.

23.2 Tablet count

Three medications of each treatment were issued in plastic bottles to the subjects with a diary card. They were required to fill the diary card in their house after the consumption of the drug on each day. A count of returned tablets was performed in the subsequent visits. Later on all unused medication were accounted for.

24.0 CLINICAL SAFETY MEASUREMENTS

Vital signs of oral temperature, sitting blood pressure and radial pulse were measured during subject check-in, prior to each dosing and two hours, six hours after administration of study drug and before checkout in each period. Vital signs to be measured prior to administration of the dose were taken within one hour of the scheduled dosing time. At all other times, vital signs were taken within 30 minutes of the scheduled times.

Brief clinical examination of the subject was conducted by a qualified medical designate on duty after subject check-in, prior to dosing of study drug and before checkout.

24.1 Handling of Safety Parameters

• Adverse Events

Subjects were monitored throughout the study period for adverse events. Subjects were informed to bring to the notice of the nurse or the doctor.
any adverse event that may occur during their stay at the site of investigation.

25.0 STATISTICAL ANALYSIS

Statistical analysis was performed on the data from subjects. If necessary, an unequal number of subjects per sequence were used. A paired-difference t-test was used at alpha = 0.05 level of significance to test for significant differences between Day 1 and Day 5. A p-value < 0.05 in the t-statistic will indicate significant difference between Day 1 and Day 5.

A one-way ANOVA was performed to assess differences between the treatment groups {(A,B,C,D,E,F,G)} based on the differences of Day 1 and Day 5 for each treatment group. A p-value < 0.05 for the F-statistic in the One-Way ANOVA indicates that at least one pair of treatment groups differ significantly. To test exactly which pair/pairs of treatment groups differed significantly, a multiple comparison test like the paired-difference t-test is required to be performed.
FIG. MM 1: SCHEMATIC REPRESENTATION OF DEFINITIVE STUDY DESIGN

Evaluation Of Combination Diuretic Therapy In Combating Furosemide Resistance In Healthy Human Volunteers Schematic Representation Of Study Design

Period I-V (n =15)

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-24hr -16 hr -12hr 1hr 0.0hr 2hr 4hr 6hr 9hr Blood sample, Body weight measurement, Arterial SBP & DBP,

Drug

24hr prior urine collection Urine Collection Vitals & checks out, ADE

ADE= Adverse Drug Events

TREATMENTS
A= Furosemide (40mg)
B= Furosemide (80mg)
C= Hydrochlorothiazide (50mg)
D= Spironolactone (50mg)
E= Furosemide (40mg) + Hydrochlorothiazide (50mg)
F= Furosemide (40mg) + Spironolactone (50mg)
G= Placebo
FIG. MM 2: SCHEMATIC REPRESENTATION OF STUDY DESIGN
To Investigate The Effect Of Multiple Dose Furosemide And Placebo
On Urinary Electrolytes And Urine Volume.

Period 1 & 2 (n = 6)

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-24hr - 16 hr -12hr 1hr 0.0hr 0hr 2hr 4hr 6hr

Check-in & vitals Drug Vitals, ADE & Check-out

24th prior urine collection Urine Collection

ADE = Adverse Drug Events
A = Furosemide (40mg)
B = Placebo
RESULTS
 RESULTS

I ETHICAL COMMITTEE APPROVAL

The study was presented to the Jamia Hamdard Institutional Review Board and the protocol, informed consent form and volunteer compensation were approved in the meeting held on 7th March 2002.

II PILOT STUDY

1.0 Subjects

Six healthy male volunteers aged 23 to 36 years (mean age 27.66 years), were selected for the study (Table No RS-1). They were screened within 28 days of the initiation of the study and underwent a full medical examination, ECG, routine laboratory tests (including hematology, blood biochemistry, urine analysis and immunological tests) in order to confirm and document that they are eligible for the study. All subjects were non-alcoholic and no nicotine or caffeine consumption was allowed on study days. None of the subjects were taking any concomitant drugs likely to interfere with the study medication. The nature of the study was explained to each of the participants and each volunteer signed an informed consent form prior to participation. All subjects completed the two periods of study. No drug related adverse events have been reported/observed during study.

Actual values are expressed as mean of day 1, day 3 and day 5. The comparison between day 1 & day 3 and day 1 & day 5 was done separately for each of the treatment groups and expressed as mean difference between day 1 & day 3 and day 1 & day 5 ± Standard deviation in respective tables. A paired difference t-test was used at alpha = 0.05 level of significance to test for significant differences between day 1 and day 5. A p value < 0.05 in the t-statistic will indicate significant difference between day 1, day 3 and day 5. A one-way ANOVA was performed to assess differences between and within the treatment groups.
Table RS 1: Demographic Details of Subjects participating in the Pilot Study

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Mean 27.66 164.166 59
SD 4.68 4.95 4.29
based on the differences of day 1, day 3 and day 5 for each treatment group.

2.0 Assessments

1. Urine volume output

Furosemide (40 mg) caused an approximately 3 fold increase in urinary output on day 1 when compared to placebo. Also 2 fold increase by furosemide was observed on day 3 in comparison to the placebo. There was significant decrease in urine volume output by furosemide multiple dosing on subsequent days 3 and 5 when compared to day 1. Mean of difference between day 1 and day 3 was (-) 851.66 ± 638.47 (p value < 0.02). In case of comparison between day 1 and day 5, the mean of difference was further increased to (-) 1111.66 ± 956.86 (p value < 0.03). In contrast to this, five days multiple dosing of placebo did not effect much of the change in urine volume output, when a comparison was made for day 1 to day 3 and day 5. In fact there was a slight increase in urine volume output on subsequent days (day 3 and day 5). However, this increase was not statistically significant (Table RS 2; Fig RS 1).

2. Urine sodium output:

There was approximately six and five fold increase in urinary sodium output on day 1 and 3 respectively of furosemide treatment when compared with the placebo. There was a decrease in urinary sodium output on subsequent days (3 and 5) of furosemide (40 mg) dosing when compared to the day 1. The mean of difference between day 1 and day 3 was (-) 46.27 ± 112.59. However, this change in sodium output was not statistically significant. There was further reduction in sodium output on day 5. The mean of difference between day 1 and day 5 was 179.16 ± 130.85 (p ≤ 0.02). In placebo there was no significant change in mean of difference between day 1 and day 3 or day 5 (Table RS 3; Fig RS 2).
Table RS 2: Mean Change in Urine volume output (ml/6hrs) following the administration of furosemide and placebo for 5 Days

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<td>from day 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>638.47</td>
<td>956.86</td>
</tr>
<tr>
<td>SEM</td>
<td>260.65</td>
<td>390.67</td>
</tr>
<tr>
<td>t-value</td>
<td>3.26</td>
<td>2.84</td>
</tr>
<tr>
<td>p-value</td>
<td>0.02*</td>
<td>0.03*</td>
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</tbody>
</table>

*= p<0.05 (Statistically significant change)

(-) ve = decrease on day 5 compared to day 1; (+) ve = increase on day 5 compared to day 1
Fig RS 1. Mean Change in Urine volume output (ml/6hrs) following the administration of furosemide and placebo for 5 Days

- Day 3
  - Furosemide
  - Placebo

- Day 5
  - Furosemide
  - Placebo

☆ = P<0.05 (STATISTICALLY SIGNIFICANT CHANGE)
<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Furosemide</td>
<td>+1.78</td>
<td>+1.79</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Furosemide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table RS: Mean change in urinary sodium output (mmol/24h) following the administration of Furosemide vs. Placebo for 5 Days.

RESULTS
Fig RS 2. Mean Change in Urinary sodium output (meq/6hrs) following the administration of furosemide and placebo for 5 Days

★ = p < 0.05 (Statistically significant change)
3. Urinary potassium output

There was an increase in urinary potassium output on furosemide (40 mg) treatment when compared to placebo. Five days multiple dose of furosemide (40 mg) did not cause significant decrease in potassium on day 3 and day 5 when compared to day 1. Similarly no significant change in urinary potassium output was noted on multiple dosing of placebo (Table RS 4; Fig RS 3).

III DEFINITIVE STUDY

1.0 Subjects

Sixteen healthy volunteers, aged 19-40 years (mean age 24.71 years), participated in study (Table RS-5). They were screened within 28 days of the initiation of the study and underwent a full medical examination, an ECG, routine laboratory tests (including hematology, blood biochemistry, urine analysis and immunological tests) in order to confirm and document that they are eligible for the study. All subjects except five were non-alcoholics subjects. Nos. 3, 6, 7, 11, 13 consumed alcohol before the screening for the study. All subjects were asked to avoid alcohol for the entire duration of the study. No nicotine or caffeine consumption was allowed on study days. The risks, burdens, benefits of the study and the commitment of the volunteers during the duration of the study were explained to each of the participants and each volunteer signed an informed consent form prior to participation. Two subjects dropped out of the study after first visit. Their demographics are not included. Two subjects replaced them in next period. Subject No. 7 was withdrawn out of the study before dosing in seventh period on the medical ground. He was having bilateral diffuse ronchi and crepts in chest, cough and pain in throat. He was referred to medicine OPD for treatment. Adverse events experienced by the subject due to non-drug factors during the entire study period have been detailed at the end. No drug related serious adverse events had been reported/observed during the study.
Table RS 4: Mean Change in Urinary potassium output (meq/6hrs) following the administration of furosemide and placebo for 5 Days

<table>
<thead>
<tr>
<th></th>
<th>Furosemide (40 mg)</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 3</td>
</tr>
<tr>
<td>N</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Mean</td>
<td>27.02</td>
<td>23.62</td>
</tr>
<tr>
<td>SD</td>
<td>12.44</td>
<td>7.65</td>
</tr>
<tr>
<td>Mean difference from day 1</td>
<td>-3.4</td>
<td>-1.72</td>
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<tr>
<td>SD</td>
<td></td>
<td>7.59</td>
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<tr>
<td>SEM</td>
<td>2.28</td>
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<td>t-value</td>
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<td>0.32</td>
</tr>
<tr>
<td>p-value</td>
<td>0.32</td>
<td>0.7585</td>
</tr>
</tbody>
</table>

*= p<0.05 (Statistically significant change)

(-) ve = decrease on day 5 compared to day 1; (+) ve = increase on day 5 compared to day 1
Fig RS 3. Mean Change in Urinary potassium output (meq/6hrs) following the administration of furosemide and placebo for 5 Days
Actual values are expressed as mean of day 1 and day 5. The comparison between day 1 and day 5 was done separately for each of the treatment groups and expressed as mean difference between day 1 and day 5 ± Standard deviation in respective tables. A paired difference t-test was used at alpha = 0.05 level of significance to test for significant differences between day 1 and day 5. A p value < 0.05 in the t-statistic will indicate significant difference between day 1 and day 5. A one-way ANOVA was performed to assess differences between the treatment groups based on the differences of day 1 and day 5 for each treatment group. So in the one-way ANOVA model, the parameter tested was difference between day 1 and day 5 for each of parameters.

A p value less than 0.05 for the F-statistic in the one way ANOVA indicates that at least one pair of treatment group differ significantly. To test exactly which pair/pairs of the treatment groups differed significantly, a multiple comparison test like the paired difference t-test was required to be performed. P value was given wherever the difference between groups was statistically significant. All statistical analysis was performed using the SAS software, version 8.1.

3.0 ASSESSMENTS:

A. Urinary Parameters

1. Volume of urine

There was approximately two fold increase in the mean urine volume output in case of furosemide (40 mg), furosemide (80 mg), furosemide (40 mg) + hydrochlorothiazide (50 mg) as compared to the placebo. All the treatment, except placebo and furosemide (40 mg) + spironolactone (50 mg), produced a statistically significant decrease in urine volume output on day 5 as compared to the day 1. Maximum decrease of mean difference of day 1 and day 5 was seen in the case of furosemide (40 mg) + hydrochlorothiazide (50 mg) (-) 660.38 ± 802.86 table RS 6, Fig RS 4). Spironolactone produced the least mean difference.
of day 1 and day 5 (175 ± 277.98 mL; p = 0.42). This was a significant decrease in urine volume output on day 5 when compared to day 1. In placebo, mean difference between day 1 and day 5 was (-) 245.7 ± 552.51 which was not statistically significant (p ≥ 0.05).

Mean difference of day 1 and day 5 of urine volume output was not statistically significant when compared between treatments. p value for the f-statistic of one-way ANOVA was 0.1382. Hence the treatment groups did not differ significantly.

2. Urinary sodium output for 12 hours

In this parameter, there was not statistically significant difference between the treatments with regard to mean difference of day 1 and day 5 (p value > 0.05).

There was approximately two fold increase in mean urinary sodium output in furosemide (40 mg), furosemide (80 mg), furosemide (40 mg) + hydrochlorothiazide (50 mg) as compared to the placebo group (Table RS 7, Fig RS 5). There was slight increase in urinary sodium output with hydrochlorothiazide (50 mg) and furosemide (40 mg) + hydrochlorothiazide (50 mg). Whereas in case of spironolactone, the value are almost comparable to placebo.

There was statistically significant decrease in the urinary output of sodium on day 5 after five days’ treatment with furosemide (40 mg). Mean difference of day 1 and day 5 in this case was (-) 133.97 ± 222.53 mL (p = 0.05). With increased dose of furosemide i.e. 80 mg, the mean difference of day 1 and day 5 further statistically significant increased 244.73 ± 244.6 (p = 0.003). A decrease in day 5 sodium output was seen in all the rest of treatment but mean difference of day 1 and day 5 was not statistically significant. Least mean difference was observed in case of furosemide (40 mg) + spironolactone (50 mg) (36.9 ± 305.81 mL)
### Table RS 6: Mean Change in Volume of Urine Output (ml/12 hrs) following the administration of Seven Treatments for 5 Days

<table>
<thead>
<tr>
<th></th>
<th>F (40mg)</th>
<th>F (80mg)</th>
<th>HCTZ (50mg)</th>
<th>S (50mg)</th>
<th>F (40mg) + HCTZ (50mg)</th>
<th>F (40mg) + S (50mg)</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>12</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>Day 1</td>
<td>Day 5</td>
<td>Day 1</td>
<td>Day 5</td>
<td>Day 1</td>
<td>Day 5</td>
<td>Day 1</td>
</tr>
<tr>
<td></td>
<td>486.9</td>
<td>891.0</td>
<td>086.1</td>
<td>306.2</td>
<td>936.1</td>
<td>761.5</td>
<td>3100.4</td>
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<td>2</td>
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<td>2</td>
<td>7</td>
<td>15</td>
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<td>828.0</td>
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<td><strong>Mean difference between day 1 &amp; day 5</strong></td>
<td>-595.77</td>
<td>-481.54</td>
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<td>-175.52</td>
<td>-660.38</td>
<td>-315.06</td>
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<td><strong>SD</strong></td>
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<td>637.72</td>
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<td>720.21</td>
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<td>0.0118*</td>
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</tbody>
</table>

F (40 mg) = Furosemide 40mg; F (80mg) = Furosemide 80mg; HCTZ (50mg) = Hydrochlorothiazide 50mg; S (50mg) = Spironolactone 50mg

* = p<0.05 (Statistically significant change)

(-) ve = decrease on day 5 compared to day 1;
### RESULTS

**Table RS 5: Demographic Details Of Subjects Participating in the Definitive Study**

<table>
<thead>
<tr>
<th>Sub No.</th>
<th>Age (Years)</th>
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<th>Weight (Kgs)</th>
<th>Alcohol Intake</th>
<th>Date of Screening</th>
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<td>79</td>
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</tr>
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<td>2</td>
<td>22</td>
<td>167</td>
<td>58</td>
<td>//</td>
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</tr>
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<td>35</td>
<td>170</td>
<td>65</td>
<td>No</td>
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</table>

| Mean    | 24.71       | 165.21      | 56.64        |                |                  |
| SD      | 5.96        | 4.64        | 9.62         |                |                  |
Fig RS 4. Mean Change in Volume of Urine Output (ml/12 hrs) following the administration of Seven Treatments for 5 Days

F 40 mg  F 80 mg  HCTZ 50mg  S 50 mg  F 40 mg + HCTZ 50mg  F 40 mg + S 50mg  Placebo

Mean difference between day 1 & day 5

F = Furosemide; HCTZ = Hydrochlorothiazide; S = Spironolactone

★ P<0.05 (STATISTICALLY SIGNIFICANT CHANGE)
### RESULTS

#### Table RS 7: Mean Change in Urinary Sodium Output (meq/12 hrs) following the administration of Seven Treatments for 5 Days

<table>
<thead>
<tr>
<th></th>
<th>F (40mg)</th>
<th>F (80mg)</th>
<th>HCTZ (50mg)</th>
<th>S (50mg)</th>
<th>F (40mg) + HCTZ (50mg)</th>
<th>F (40mg) + S (50mg)</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
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<td>13</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>530.9</td>
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<td>378.28</td>
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<td>199.61</td>
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<tr>
<td>Day 5</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>94.94</td>
<td>595.01</td>
<td>350.28</td>
<td>378.28</td>
<td>271.19</td>
<td>270.4</td>
<td>199.61</td>
<td>272.0</td>
</tr>
<tr>
<td>Mean difference between day 1 &amp; day 5</td>
<td>-133.97</td>
<td>-244.73</td>
<td>-107.087</td>
<td>-70.785</td>
<td>-241.52</td>
<td>-36.9</td>
<td>-75</td>
</tr>
<tr>
<td>SD</td>
<td>222.53</td>
<td>244.6</td>
<td>309.65</td>
<td>132.97</td>
<td>458.67</td>
<td>305.81</td>
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<td>SEM</td>
<td>61.719</td>
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<td>12</td>
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<td>t-value</td>
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<td>p value</td>
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</table>

F (40 mg) = Furosemide 40mg; F (80mg) = Furosemide 80mg; HCTZ (50mg) = Hydrochlorothiazide 50mg; S (50mg) = Spironolactone 50mg

*= p<0.05(Statistically significant change)

(-) ve = decrease on day 5 compared to day 1;
Fig RS 5. Mean Change in Urinary Sodium Output (meq/12 hrs) following the administration of Seven Treatments for 5 Days

F 40 mg  F 80 mg  HCTZ 50mg  S 50 mg  F 40 mg + HCTZ 50mg  F 40 mg + S 50mg  Placebo

Mean difference between day 1 & day 5

F = Furosemide; HCTZ = Hydrochlorothiazide; S = Spironolactone

☆ = p<0.05 (Statistically significant change)
3. Urinary potassium output

There was a two-fold increase in mean urinary potassium output with furosemide (40 mg) + hydrochlorothiazide (50 mg) treatment on both days (day 1 and day 5) when compared with the placebo. Least mean difference in potassium output was seen in the spironolactone (50 mg) treatment group.

There was statistically significant decrease in the urinary output of potassium on day 5 after five days of treatment with furosemide (80 mg) (Mean difference between day 1 and day 5 = 14.81; p = 0.0297). Hydrochlorothiazide and placebo also showed a reduction in urinary output on day 5 as compared to day 1 as mean difference of day 1 and day 5 of (-) 12.60 ± 18.67 (p < 0.05) and (-) 13.697 ± 19.928 (p = 0.0291) respectively. In contrast to this, it was seen that furosemide (40 mg) and spironolactone produce slight increase in the potassium output after 5 days of multiple dosing which was not statistically different from day 1. In case of furosemide mean difference between day 1 and day 5 was 7.611 ± 38.646 whereas for spironolactone it was 8.202 ± 19.74 (Table RS 8; Fig RS 6).

There was no statistically significant difference between the treatment with regard to mean of difference between day 1 and day 5 (p value > 0.05).

B. Serum electrolytes at 12 hours post dose

1. Serum sodium concentration

In all the treatment mean values for serum sodium concentration 12 hour post dose was within normal physiological range on day 1 and day 5 of the 5 days treatment regime. No comparisons were statistically significant either at within or between the treatments. However, most of the treatment groups except furosemide (40 mg) and furosemide (40 mg) + spironolactone (50 mg) produced an increase in serum sodium at 12
Table RS 8: Mean Change in Urinary Potassium Output (meq/12 hrs) following the administration of Seven Treatments for 5 Days

<table>
<thead>
<tr>
<th></th>
<th>F (40mg)</th>
<th>F (80mg)</th>
<th>HCTZ (50mg)</th>
<th>S (50mg)</th>
<th>F/(40mg) + HCTZ (50mg)</th>
<th>F (40mg) + S (50mg)</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
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<td>13</td>
<td>13</td>
<td>13</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Mean</td>
<td>Day 1</td>
<td>Day 5</td>
<td>Day 1</td>
<td>Day 5</td>
<td>Day 1</td>
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<td>51.02</td>
<td>37.09</td>
<td>24.48</td>
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</tr>
<tr>
<td>between day 1 &amp; day 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>38.646</td>
<td>30.32</td>
<td>18.67</td>
<td>19.74</td>
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<td>p value</td>
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<td>0.16</td>
<td>0.2836</td>
<td>0.135</td>
<td>0.0291*</td>
</tr>
</tbody>
</table>

F (40 mg) = Furosemide 40mg; F (80mg) = Furosemide 80mg; HCTZ (50mg) = Hydrochlorothiazide 50mg; S (50mg) = Spironolactone 50mg

*= p<0.05 (Statistically significant change)

(-) ve = decrease on day 5 compared to day 1; (+) ve = increase on day 5 compared to day
Fig RS 6. Mean Change in Urinary Potassium Output (meq/12 hrs) following the administration of Seven Treatments for 5 Days

F = Furosemide; HCTZ = Hydrochlorothiazide; S = Spironolactone  ★ = p<0.05 (statistically significant change)
hour post dose on day 5 as compared to day 1 which of course were not statistically significant (Table RS 9; Fig RS 7).

2. Serum potassium concentration

Mean serum potassium levels were in normal physiological level in all the treatment groups on both day 1 and day 5. There was a significant decrease in serum potassium concentration at 12 hour post dose on day 5 in comparison to day 1 when hydrochlorothiazide was given for five days. Mean difference of day 1 and day 5 in hydrochlorothiazide group was \(-0.2455 \pm 0.3616\) which was statistically significant. No significant changes were seen in mean differences of potassium concentration at 12 hours post dose with other treatment group (Table RS 10; Fig RS 10). Five days multiple dosing of spironolactone (50 mg), furosemide (40 mg) + spironolactone (50 mg) and placebo showed a mean of difference between day 1 and day 5 as \(0.155 \pm 0.5355, 0.14 \pm 0.7676\) and \(0.027 \pm 0.4606\). This means that there was increase in serum potassium levels on day 5 at 12 hours post dose in comparison to day 1.

In contrast to this there was decrease in serum potassium concentration 12 hour post dose on day 5 in comparison to day 1 after five days of treatment with furosemide (40 mg), furosemide (80 mg), hydrochlorothiazide (50 mg) and furosemide (40 mg) + hydrochlorothiazide (50 mg). This mean difference between day 1 and day 5 were not statistically significant.

The mean of differences between day 1 and day 5 of serum potassium concentration 12-hour post dose between the treatments were not statistically different.
Table RS 9: Mean Change in Blood Sodium (mM/L 12 hrs Post Dose) following the administration of
Seven Treatments for 5 Days

<table>
<thead>
<tr>
<th></th>
<th>F (40mg)</th>
<th>F (80mg)</th>
<th>HCTZ (50mg)</th>
<th>S (50mg)</th>
<th>F (40mg) + HCTZ (50mg)</th>
<th>F (40mg) + S (50mg)</th>
<th>Placebo</th>
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<td>Day 5</td>
<td>Day 1</td>
<td>Day 5</td>
<td>Day 1</td>
<td>Day 5</td>
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<td>41.38</td>
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<td>P-value</td>
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<td>0.8071</td>
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</tr>
</tbody>
</table>

F (40 mg) = Furosemide 40mg; F (80mg) = Furosemide 80mg; HCTZ (50mg) = Hydrochlorothiazide 50mg; S (50mg) = Spironolactone 50mg

(-) ve = decrease on day 5 compared to day 1; (+) ve = increase on day 5 compared to day
Fig RS 7. Mean Change in Blood Sodium (mM/L 12 hrs Post Dose) following the administration of Seven Treatments for 5 Days

F 40 mg  F 80 mg  HCTZ 50mg  S 50 mg  F 40 mg + HCTZ 50mg  F 40 mg + S 50mg  Placebo

F = Furosemide; HCTZ = Hydrochlorothiazide; S = Spironolactone
### Table RS 10: Mean Change in Blood Potassium (mM/L 12 hrs Post Dose) following the administration of Seven Treatments for 5 Days

<table>
<thead>
<tr>
<th></th>
<th>F (40mg)</th>
<th>F (80mg)</th>
<th>HCTZ (50mg)</th>
<th>S (50mg)</th>
<th>F (40mg) + HCTZ (50mg)</th>
<th>F (40mg) + S (50mg)</th>
<th>Placebo</th>
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<tr>
<td><strong>Mean</strong></td>
<td>Day 1</td>
<td>Day 1</td>
<td>Day 1</td>
<td>Day 1</td>
<td>Day 1</td>
<td>Day 1</td>
<td>Day 1</td>
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<tr>
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<td>Day 5</td>
<td>Day 5</td>
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<td><strong>Mean difference between day 1 &amp; day 5</strong></td>
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<td>-0.1273</td>
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<td>0.1575</td>
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</tr>
<tr>
<td><strong>t-value</strong></td>
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<td>0.5766</td>
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</table>

F (40 mg) = Furosemide 40mg; F (80mg) = Furosemide 80mg; HCTZ (50mg) = Hydrochlorothiazide 50mg; S (50mg) = Spironolactone 50mg

* = p<0.05 (Statistically significant change)

(-) ve = decrease on day 5 compared to day 1; (+) ve = increase on day 5 compared to day 1
Fig RS 8. Mean Change in Blood Potassium (mM/L 12 hrs Post Dose) following the administration of Seven Treatments for 5 Days

<table>
<thead>
<tr>
<th>Treatment</th>
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<tbody>
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<td>F 40 mg</td>
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<td>F 80 mg</td>
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<td>HCTZ 50 mg</td>
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<td>S 50 mg</td>
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<tr>
<td>F 40 mg + HCTZ 50 mg</td>
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<tr>
<td>F 40 mg + S 50 mg</td>
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<tr>
<td>Placebo</td>
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</tbody>
</table>

F = Furosemide; HCTZ = Hydrochlorothiazide; S = Spironolactone

★ = p<0.05 (Statistically significant change)
Arterial blood pressure

1. Systolic blood pressure (SBP) 12-hour post dose
   None of treatment had decreased/increased SBP above or below the normal physiological range. Mean of differences of day 1 and day 5 did not differ significantly between the treatments (p value > 0.05).
   Also none of the treatments produced significant fall or rise in the SBP on five days multiple dosing. Maximum fall of SBP was produced by the five-day treatment of spironolactone (mean of difference between day 1 and day 5 = 4). However, this fall was not statistically significant. The rise of SBP was only associated with furosemide (40 mg) + hydrochlorothiazide (50 mg) treatment group (Table RS 11; Fig RS 9).

2. Diastolic blood pressure (DBP) 12 hour post dose
   No treatment had affected the DBP so that it falls or rises outside the normal physiological range. There was a very slight variation in the change in DBP. No comparisons were statistically significant either at within or between treatments. Maximum fall in DBP was associated with five day treatment of spironolactone (mean of difference between day 1 and day 5 was 3.84). The rise in DBP was only associated with the treatment of furosemide (40 mg) + hydrochlorothiazide (50 mg). Mean of difference between day 1 and day 5 was -2.33 and p value was 0.1891 which is greater than 0.05 (Table RS 12; Fig RS 10).

C. Body weight 12 hour post dose
   There was a very slight variation in the change in body weight. No comparisons between the treatments were statistically significant. Mean difference of body weight between day 1 and day 5 was statistically significant in spironolactone treatment for five days (mean of difference between day 1 and day 5 = 0.3631; p value = 0.0174). However, in all the other treatment, there was no significant difference between day 1 and day 5 body weight.
Table RS 11: Mean Change in Systolic Blood Pressure (mmHg12 hrs, Post Dose) following the administration of Seven Treatments for 5 Days

<table>
<thead>
<tr>
<th></th>
<th>F (40mg)</th>
<th>F (80mg)</th>
<th>HCTZ (50mg)</th>
<th>S (50mg)</th>
<th>F (40mg) + HCTZ (50mg)</th>
<th>F (40mg) + S (50mg)</th>
<th>Placebo</th>
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</tr>
<tr>
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<tr>
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F (40 mg) = Furosemide 40mg; F (80mg) = Furosemide 80mg; HCTZ (50mg) = Hydrochlorothiazide 50mg; S (50mg) = Spironolactone 50mg

(-) ve = decrease on day 5 compared to day 1; (+) ve = increase on day 5 compared to day
Fig RS 9. Mean Change in Systolic Blood Pressure (mmHg 12 hrs, Post Dose) following the administration of Seven Treatments for 5 Days

- F 40 mg
- F 80 mg
- HCTZ 50mg
- S 50 mg
- F 40 mg + HCTZ 50mg
- F 40 mg + S 50mg
- Placebo

Mean difference between day 1 & day 5

F = Furosemide; HCTZ = Hydrochlorothiazide; S = Spironolactone
### RESULTS

**Table RS 12: Mean Change in Diastolic Blood Pressure (mmHg 12 hrs, Post Dose) following the administration of Seven Treatments for 5 Days**

<table>
<thead>
<tr>
<th></th>
<th>F (40mg)</th>
<th>F (80mg)</th>
<th>HCTZ (50mg)</th>
<th>S (50mg)</th>
<th>F (40mg) + HCTZ (50mg)</th>
<th>F (40mg) + S (50mg)</th>
<th>Placebo</th>
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<td>Mean</td>
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<td>Day 5</td>
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<td>Day 1</td>
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</table>

F (40 mg) = Furosemide 40mg; F (80mg) = Furosemide 80mg; HCTZ (50mg) = Hydrochlorothiazide 50mg; S (50mg) = Spironolactone 50mg

(⁻) ve = decrease on day 5 compared to day 1; (+) ve = increase on day 5 compared to day

90
Fig RS 10. Mean Change in Diastolic Blood Pressure (mmHg 12 hrs, Post Dose) following the administration of Seven Treatments for 5 Days

- F 40 mg
- F 80 mg
- HCTZ 50mg
- S 50 mg
- F 40 mg + HCTZ 50mg
- F 40 mg + S 50mg
- Placebo

Mean difference between day 1 & day 5

F = Furosemide; HCTZ = Hydrochlorothiazide; S = Spironolactone
**Table RS 13: Mean Change in Body Weight (Kg 12 hrs Post Dose) following the administration of Seven Treatments for 5 Days**

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<th>F (40mg)</th>
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<th>HCTZ (50mg)</th>
<th>S (50mg)</th>
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<td>Day 1</td>
<td>Day 1</td>
<td>Day 1</td>
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<td>Day 1</td>
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<td>1 &amp; day 5</td>
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<td>1 &amp; day 5</td>
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<td>0.658</td>
<td>0.6058</td>
<td>0.5221</td>
</tr>
</tbody>
</table>

F (40 mg) = Furosemide 40mg; F (80mg) = Furosemide 80mg; HCTZ (50mg) = Hydrochlorothiazide 50mg; S (50mg) = Spironolactone 50mg

* = p<0.05 (Statistically significant change)

(-) ve = decrease on day 5 compared to day 1; (+) ve = increase on day 5 compared to day 1
Fig RS 11. Mean Change in Body Weight (Kg 12 hrs Post Dose) following the administration of Seven Treatments for 5 Days

- F 40 mg
- F 80 mg
- HCTZ 50mg
- S 50 mg
- F 40 mg + HCTZ 50mg
- F 40 mg + S 50mg
- Placebo

Mean difference between day 1 & day 5

- F = Furosemide; HCTZ = Hydrochlorothiazide; S = Spironolactone
- ★ = p<0.05 (Statistically significant change)
DISCUSSION


Discussion

Diuretics are widely used in cardiovascular medicine for several decades. They are cornerstone treatment line for all forms of congestive heart failure and are formally indicated in hypertension accompanied by congestive heart failure or renal insufficiency.

Diuretic drugs are usually effective treatment for edema when used judiciously. However, some patients become resistant to their effect. When a diuretic drug is administered, rates of urinary sodium and chloride excretion usually increase above baseline leading to a period of negative sodium and chloride balance. These changes in diuretic responsiveness result from adaptive processes that occur during diuretic therapy. When these processes become manifest once the desired extra cellular fluid (ECF) volume has been attained, they are clinically useful and prevent progressive ECF volume contraction. When these processes develop prior to achieving the desired ECF volume, they can be viewed as contributing to diuretic resistance (Ellison, 1999).

Diuretic resistance involves both pharmacokinetic and pharmacodynamic factors. Pharmacokinetic factors take into account total amount of free diuretic reaching the site of action and time course of delivery into urine. Pharmacodynamic factors are not well understood and needs further research. However, most of the studies looking for the pharmacodynamic factors have focused on the adaptation that occurs on repeated administration of diuretics. Diuretic adaptations are classified as those that occur during diuretic action, those that cause sodium retention in the short term (causing “post diuretics sodium retention”) and those that increase sodium retention chronically (the braking phenomena).

Recent experimental work has indicated ways in which kidney adapts itself to chronic diuretic treatment. First nephron segment downstream increase NaCl reabsorption during diuretic administration
because from the site of diuretic action delivered NaCl load is increased. Second, when diuretic concentration in the tubule decline, the kidney tubules act to retain Na until the next dose of diuretic is administered. Third, the ability of the diuretic to increase renal NaCl excretion declines over time, an effect that results both from depletion of ECF volume and from structural and functional changes of kidney. All these adaptation increase the rate of NaCl reabsorption and blunt the effectiveness of diuretic therapy.

Diuretic resistance is found to be associated with many clinical conditions as in congestive heart failure, chronic renal insufficiency, nephrotic syndrome, and cirrhosis. Many strategies have been used to overcome diuretic resistance. Many times high doses of diuretic or constant intravenous infusion are employed. Another approach is to administer more than one class of diuretic agents. Recent experimental results suggest that a second diuretic agent may act synergistically because it blocks the adaptive processes limiting to the effectiveness of first diuretic. Several studies in clinical setting have supported the synergistic nature of diuretic combination. However, most of these studies have not focused on the mechanism(s) behind this synergistic effect. It was presumed on the basis of experimental studies that patients who manifest a beneficial response are those which express the adaptive processes. Very few studies have been conducted with healthy volunteer with an aim to delineate the mechanism of this synergistic effect.

Therefore the present study was designed to evaluate the effect of chronic dosing of hydrochlorothiazide (50mg) plus furosemide (40mg) and spironolactone (50mg) plus furosemide (40mg) in comparison to furosemide (40mg), furosemide (80 mg), hydrochlorothiazide (50 mg), spironolactone (50 mg) and placebo on electrolytes, urine volume, body weight and arterial blood pressure in healthy human subjects.

We have carried out the evaluation in healthy subject with the perspective of biological homogeneity, in as much as patients with fluid
retention or impaired renal function respond in scattered manner. When evaluation of diuretics is carried out on such type of patient population, study should include large sample size and conclusions should be strictly spelled on that specified group rather than generalizing them. We also tried to develop a human model, which can give an insight of the mechanism of resistance that will help in optimizing therapy.

The intense natriuresis that follows oral administration of a loop diuretic is usually complete in about three to six hours after dosing. As a result, mean overall natriuresis after 6 hours is less than what is expected. This is due to mean rebound or undershoots natriuresis. Single oral doses of thiazide type diuretic increase natriuresis more slowly than single oral doses of loop diuretic. However overall natriuresis during 6 – 24 hour period after dosing with a thiazide diuretic does not fall. The rebound in urinary sodium excretion that occurs after oral administration of single dose of loop diuretic is principally due to increased nephronal tubular reabsorption of filtered sodium. This is because of functional and structural adaptive changes, which occur in the proximal convoluted tubule, early distal tubule and in late tubular structure. Therefore we have combined a diuretic agent that act at distal convoluted tubule and another at collecting tubule in order to see the synergism and recognize the site of synergism by sequential nephron blockade in healthy human volunteer.

Most of the principles envisaged in the ICH: Good Clinical Guidelines (1996) were followed. The study protocol, informed consent form, and the volunteer compensation were approved by the Institutional Review Board of Jamia Hamdard (Hamdard University). The benefits, burden, and risks of their participation in the study were explained to the subjects and written informed consent was taken prior to their entry into trial. One signed copy of the consent form was given to subjects and the other copy was retained with us for documentation. The standard
SOPs of the Ranbaxy Clinical Pharmacological Unit were adhered to while conducting the study.

Statistical analysis software (SAS) was used for generating the Randomization schedule.

In this study due importance was given and various methods (counseling, pill count, diary card) were employed to attain the maximum possible compliance level.

Counseling: Each subject was counseled to take tablets on each day to meet the objective of the study and was told to contact the investigator for any clarification on any of the day. They were told about the planned pooling of data for analysis and discrepancy in the results, if they don’t consume the medication as prescribed.

Pill count: The number of tablets on each day. They were supposed to enter date and time of self-administration and any adverse even experienced. This diary card entry also acts as a remainder for the subjects to take the medication.

As the study involved multiple dosing, subjects were asked to take tablet orally in their home itself, in a designated way

Pilot study
The objective of the pilot study was to validate the phenomena of development of resistance in healthy human volunteer by evaluating the effect of repeated doses of furosemide on urinary electrolyte and urine volume. For the validation, furosemide (40 mg) once daily was administered for five days and effects are compared against the placebo treatment, which is also given for five days. The reason for giving the drug for five days is based on previous experiments done to study early phase of adaptation associated with diuretic therapy. Administration of furosemide 40 mg in normal volunteers have shown that resistance to natriuretic response develops after three days of treatment (Wilcox et al., 1983)
The study was randomised, single blind, two treatment, two period, crossover pharmacodynamic study to evaluate the effects of multiple dose furosemide (40 mg) and placebo on the urinary electrolytes and urinary volume, in six healthy, adult, male, human subjects under fasting conditions. Urine collection was by spontaneous voiding on Day1, Day3 and Day5. On the basis of the pilot study, the methodology and number of days for which the furosemide will be given in definitive study was decided. Data from day1 has provided the baseline value for comparison with day3 and day5. Collection of urine was done at predetermined interval for six hours. Fluid replacement was given by providing equivalent amount of water to drink to each volunteer after every urination. The main intention behind this was to maintain a neutral water load of the body and to prevent dehydration which could have occurred because of profused diuresis. Subject were particularly instructed and motivated to adhere to their routine pattern of diet for whole duration of study so that dietary factors should not confound the results of the study.

In the present study furosemide 40 mg has caused profused diuresis after single oral dose which on repeated once daily administration for five days has exhibited a gradual decrease. The decrease was significant on day3 with further reduction on day5. On day5, the diuresis was reduced to such an extent that it was almost comparable to the placebo response. The results obtained in the present study are in agreement with observation that furosemide on repeated administration in patients fails to reduce edema and the increase in urinary water excretion.(Gerlag and Dorman, 1996)

The diuretic potency of an oral diuretic is defined and assessed through the natriuretic action of these drugs. There was a decrease in the intensity of the natriuresis induced by the furosemide in our study when given over a period of five days. Although the decrease in natriuresis was present on day3 but statistically significant reduction
was observed only after five days of administration of drug. Wilcox et al (1983) found a significant decrease in natriuresis after three days of treatment in salt restricted group. Salt restriction was not used in the present study because of the inherent impracticability of enforcing in our outpatient based study. This may be the reason for the nonsignificant decrease in natriuresis observed after 3 days of diuretic treatment.

Potassium filters from the glomerulus and it undergoes reabsorption from the tubular fluid and excretion into it along the microtubular renal system. It is reabsorbed in the thick ascending limb of the loop of Henle together with the chloride and sodium. Kaliuretic effect of loop diuretic is linearly proportional to natriuretic effect. The early after-dosing increase in kaliuresis that follows intake of single dose of a high potency of loop diuretic is not followed by important potassium conservation after six hours of diuretic administration. This is at variance with the significant reduction in natriuresis, chloruresis and water excretion that takes place with the repeated dosing of loop diuretic. In the present study although there was increase in potassium output after single dose furosemide treatment but there was no change in potassium output after the repeated dosing of furosemide. However it is reported in literature that the elevation of mean 24 hour kaliuresis caused by the first dose of loop diuretic becomes attenuated in course of once daily treatment of furosemide (Reyes and Taylor, 1999). This is due to a relevant part of the sodium whose absorption in the thick ascending loop of Henle is impeded by loop diuretic is reabsorbed in the early distal tubule, together with the chloride and water. As a consequence, sodium and flow rate are relatively decreased in the even more distal tubular structure where potassium is excreted in response to augmentation of these variable. The contrast results in our study could be because either the dose of furosemide was low or the number of days for which furosemide was administered were insufficient to elicit resistance. In our
definitive study furosemide 80 mg showed a significant decrease in kaliuresis when administered over a period of five days.

On the basis of results obtained in the pilot study, tolerance to urinary volume and electrolyte excretion was observed when furosemide is administered over five days. This methodology was therefore, used in the definitive study with furosemide administration for five days.

Definitive Study

The primary objective of the study was to evaluate in healthy subject, the multiple dose effects of furosemide (40mg) plus hydrochlorothiazide (50mg) and furosemide (40mg) plus spironolactone (50mg) combinations in comparison to furosemide (40mg), furosemide (80mg) hydrochlorothiazide (50mg), spironolactone (50 mg) and placebo on the electrolytes and urinary volume.

The secondary objective of the study was to evaluate the effect of above-mentioned interventions on the body weight and arterial blood pressure if any.

The study was conducted in randomised, single blind, crossover, placebo controlled, seven treatment, seven period, multiple dose pharmacodynamic study. Urine collection was by spontaneous voiding on Day1 and Day5. Data from day1 has provided the baseline value for comparison of day5. Collection of urine was done at predetermined interval for twelve hours. Fluid replacement was given by providing equivalent amount of water to drink to each volunteer after every urination. Subject were particularly instructed and motivated to adhere to routine pattern of diet for whole duration of study.

Diuretic- induced increase in urine volume

There was a significant reduction in the urine volume on day5 as compared to day1 when furosemide 40 mg was administered over five days repeatedly. Similar response was observed with the higher dose of furosemide that is 80 mg. Infact all the treatment except, furosemide 40 mg plus spironolactone 50 mg, showed a significant decrease in urine
volume on day 5, as compared to day 1. Resistance to furosemide involves functional and structural adaptive changes. Functional adaptive changes involves complex interplay of several factors including sympathetic renal nerve activity, renin angiotensin aldosterone axis, vasopressin and atrial natriuretic peptide secretion (Kelly et al., 1983; Jesperssen et al., 1990; Peterson et al., 1991). From the results of our study, resistance in healthy volunteers seems to be more because of functional changes rather than structural adaptive changes (hypertrophy and hyperplasia of distal convoluted tubule). This is because if the latter factors were involved, the increase in diuresis which is caused by the addition of hydrochlorothiazide 50 mg to furosemide 40 mg should have been maintained for five days. The increase in diuresis in this group was less than additive as compared to the diuresis produced by these two drugs individually followed by the tolerance which is also toward the additive side. Combination of furosemide 40 mg and spironolactone 50 mg did not exhibit synergism i.e. increase in urinary output on day 1, as compared to the response to furosemide 40 mg alone. However, the decrease in diuresis on day 5 was not statistically significant. In this respect, the furosemide 40 mg and spironolactone 50 mg combination fared better than the furosemide 40 mg plus hydrochlorothiazide 50 mg combination.

As far as functional adaptive changes are concerned, from our study we are able to conclude the possible involvement of the following two factors may be involved:

Most of the diuretics stimulate renin secretion by reduction in the ECF volume. It is reported that among the various neurohumoral factors physiologically involved in response to change in sodium-water intake or excretion, the reactivity of renin angiotensin system plays a leading role (Burnier and Burnier, 1992). Hence, the reduction in diuresis seen in our study can result from activation of Renin-
Angiotensin-Aldosterone system (RAAS) by the repeated doses of the diuretics. This hypothesis is further supported by the observation that group receiving furosemide along with spironolactone for 5 days has exhibited little decrease in urine output. This may be due to blunting of actions of the elevated levels of aldosterone by spironolactone.

However, if results of the urinary sodium output are also taken into account, it was seen that addition of hydrochlorothiazide overcomes tolerance produced by the furosemide. This suggests that some other factor is also involved which is causing conservation of water but loss of sodium. When aldosterone causes increase in sodium reabsorption from tubule, this causes a simultaneous reabsorption of water and an increase in arterial pressure and the increase in arterial pressure leads to increased glomerular filtration rate. The rapid flow of filtrate down the tubular system then overrides the excessive reabsorptive effect of aldosterone thereby completely nullifies the effect of aldosterone on ECF sodium concentration. Further vasopressin thirst overshadows the aldosterone system (Guyton, 1991)

Urinary Sodium Output

From the present study, it is evident that there was a significant reduction in natriuresis during once daily treatment for 5 days with loop diuretic but not with hydrochlorothiazide. The reduction in natriuresis with furosemide was dose dependent. With the furosemide 80 mg, the mean difference of day 1 and day 5 was almost double of furosemide 40 mg. This is in agreement with the current therapeutic strategy to use high doses of diuretic in an attempt to overcome the resistance (Gerlag and VanMeijel, 1988). Maximal natriuresis was observed in combination group of hydrochlorothiazide and furosemide. However, there was some reduction in the natriuresis on day 5, which was not statistically significant. When spironolactone was added to the furosemide, there was no significant decrease in the sodium output after five days treatment. This is consistent with our hypothesis of involvement of activation of RAA
for diuretic resistance. Although most diuretic stimulate renin secretion by reducing ECF volume, only loop diuretic stimulate renin secretion at the macula densa. Thus, when ECF contraction is prevented, diuretic that acts in the DCT or collecting duct as thiazide and amiloride, have little effect on renin secretion. In contrast, under these conditions, loop diuretics strongly stimulate renin secretion (Martinez et al., 1990).

Spironolactone has shown minimal effect, which was almost equal to that observed in placebo group. This is because of facultative nature of spironolactone. The mean twenty-four our natriuresis does not increase significantly after dosing with spironolactone 100 mg in healthy individuals. This result exemplifies facultative nature of spironolactone (Reyes AJ, 1992). Since in the same study, sodium intake was high as revealed by the mean value of 24-hour natriuresis after placebo. Therefore, the RAAS axis was depressed; plasma aldosterone was not high enough to constitute a relevant determination of natriuresis and spironolactone was ineffectual. When spironolactone was co administered with hydrochlorothiazide 25 mg in the same study, the rise in aldosterone provoked by natriuretic action of early distal tubular diuretic set the stage for natriuretic effect of antialdosterone diuretic which become manifest as a potentiation of the natriuretic action of hydrochlorothiazide. Similar effects were observed in our study when furosemide was added to the spironolactone.

Urinary potassium output

There was no significant effect of repeated dose furosemide 40 mg on potassium excretion. This is consistent with the results of our pilot study. Possible reason for this could be that elevation in plasma aldosterone and vasopressin has increased kaliuresis. However, at higher doses of furosemide, there was significant reduction in the potassium output after five days of treatment. Hydrochlorothiazide 50 mg had also significantly reduced potassium excretion on repeated dosing. Kaliuresis was least marked with spironolactone on day 1. There was a
nonsignificant increase in kaliuresis on day 5 with repeated doses of spironolactone (as compared to day 1). This is consistent with the well-documented pharmacological actions of spironolactone on urinary potassium (Dolley, 1999b). Maintenance of kaliuresis is seen with the combination treatment. Kaliuresis was highest in the group receiving hydrochlorothiazide 50 mg plus furosemide 40 mg on day 1 but the effect was markedly less than additive synergism. However, on day 5 the kaliuresis was not significantly different from that produced by furosemide 40 mg alone. Hence, additive synergism was observed on day 5.

Serum Sodium and Potassium

Repeated dosing of furosemide or any other treatment had no effect on serum sodium level in healthy volunteers, despite profuse diuresis. This shows that activation of neurohumoral functional changes are intact enough to maintain the sodium serum concentration within normal physiological limits. There was a significant reduction in potassium level after repeated hydrochlorothiazide treatment. However, this reduction in potassium level is within normal physiologic range. No other treatment significantly effected potassium concentrations on repeated administration and despite profuse kaliuresis.

Arterial Blood Pressure and Bodyweight

Repeated dosing of furosemide or any other treatment had no effect on arterial blood pressure in healthy volunteer. Similarly, body weight was not significantly affected by any of treatment except spironolactone 50 mg. Spironolactone 50 mg significantly decreased the weight on day 5 as compared to day 1 but this appears to have little clinical significance.

Apart from providing the efficacy data, the secondary parameters also provide safety data of these treatments. It proves that furosemide up to the dose of 80 mg and its combination with hydrochlorothiazide & spironolactone at doses as high as 50 mg had no deleterious effect in
healthy human volunteers. Hence, if this model is to be developed further the safety concerns would be less.

**Adverse events**

All treatments were well tolerated. One subject had pain in the left eye with vague history of insect bite when he was on furosemide 80 mg. One subject had burning sensation in foot when he was on hydrochlorothiazide treatment. He was advised hot fomentation twice a day. In combination group (furosemide 40 mg plus hydrochlorothiazide 50 mg), one subject had maculopapular lesion over prepuce and burning micturition. The attending physician advised him for personal hygiene locally in genital area and norflox 400 mg BD. None of these adverse events were likely to be drug related.

**Limitation of the present study**

One of the main limitations of the present study is that, these types of studies should ideally be conducted in a metabolic unit where patients can be kept in-house for the duration of study and adequate control of dietary salt and water intake can be ensured. The high intersubject variability observed in our study is because volunteer were treated on an outpatient basis and hence, their compliance regarding dietary concerns and water intake could not be strictly monitored.

In our study, activation of RAAS and vasopressin are contributing factors to diuretic tolerance. How much these factors are intact and operative in edematous disorder would play an important role for deciding the extrapolation of this data to the disease states in which diuretics are used. Thus, the data obtained from this model needs validation in studies on actual patient. Also, we have not looked at other neurohumoral factors like ANP secretion, which will definitely seems to interrelate with the response of these factors.

In retrospect, the dosages of diuretics used in this model appear to be rather conservative, as compared to the dosages used in edematous disease states. However, the use of conservative doses in this study was
DISCUSSION

guided by the safety and ethical concerns, since participation in this study provided no therapeutic benefit whatsoever to the study subjects.

The following main conclusion could be drawn from the results of the study:

1. Repeated dose of furosemide (once daily for five days) causes significant reduction in urine volume and urinary electrolyte and thereby leads to development of tolerance to the effectiveness of furosemide treatment in healthy human volunteer.

2. The development of resistance to the diuretic effect of furosemide is dose proportional as the reduction in natriuresis induced by repeated dose of furosemide 80 mg was almost double of furosemide 40 mg.

3. Combination of furosemide with a diuretic acting on distal tubules like hydrochlorothiazide 50 mg, increased diuretic response. However, it was less than additive in comparison to situations where two are given individually.

4. Combination of furosemide with a diuretic, which is an antagonist of aldosterone like spironolactone, counteracted the resistance and diuresis was maintained even after repeated dosing of furosemide. This combination proved to be best bet, from among all the treatments studied.

5. Functional adaptive changes (Activation of RAA and vasopressin secretion) are the major contributing factors for development of resistance in healthy human volunteers.

6. Large inter-individual variations with time were seen in this outpatient based study. This may be due to lack of strict voluntary control on dietary electrolytes and water intake, despite our best efforts. Replication of this study in normal volunteers admitted in a metabolic unit is therefore warranted to refine this model further.