International Journal of Pharmaceutics, 11 (1982) 237–247
Elsevier Biomedical Press

The analysis of 4-chloro-5-sulfamoylanthranilic acid in the bulk material and pharmaceutical preparations of furosemide by a high-performance liquid chromatographic method

R.S. Rapaka, J. Roth and V.K. Prasad

Biopharmaceutics Laboratory Branch, Division of Biopharmaceutics, Food and Drug Administration (HFD-524), Washington, DC 20204 (U.S.A.)

(Received August 21st, 1981)
(Modified version received January 25th, 1982)
(Accepted January 26th, 1982)

Summary

The current USP procedure for the limit tests for 4-chloro-5-sulfamoylanthranilic acid (CSA) in furosemide preparations is a tedious and non-specific method. A simple, sensitive and specific method for the quantitative analysis of CSA from bulk materials and pharmaceutical preparations is described. The procedure consists of extraction into 0.1 N sodium hydroxide solution, dilution, addition of internal standard and injection onto a high-performance liquid chromatograph. The eluate is monitored by ultraviolet absorption at 280 nm. Utilizing this procedure, CSA was quantitatively analyzed from furosemide tablets, furosemide injection and furosemide bulk powder.

Introduction

4-Chloro-5-sulfamoylanthranilic acid (CSA) is either a degradation product or contaminant in the bulk powder and other pharmaceutical preparations, containing 4-chloro-5-furfuryl-5-sulfamoylanthranilic acid (furosemide). Hence the USP has imposed (United States Pharmacopeia XX, 1980) limit tests for CSA and the USP monographs that require a limit test are, furosemide USP, furosemide tablets USP and furosemide injection USP. The current USP procedure consists of diazotization, coupling to N-1-naphthylethylenediamine hydrochloride, followed by quantitation by UV measurement. The procedure involves utilization of acidic conditions in the

0378-5173/82/0000-0000/$02.75

diazotization step and furosemide is known to be degraded (Yakatan et al., 1976; Smith et al., 1980) in the presence of strong acid. Moreover, this procedure is time consuming and non-specific.

The first quantitative method for the analysis of CSA in urine was described by Haussler and Hajdu (1964). The main disadvantages of this method were the laborious procedure and lack of specificity. Later a number of TLC procedures appeared in literature for quantitative estimation of CSA from plasma and/or urine. Yakatan et al. (1976) described a TLC procedure for CSA and furosemide using radioactively labelled compounds in their metabolic studies on furosemide. A TLC procedure was described by Mikkelsen and Andreasen (1977) for the simultaneous determination of furosemide, anthranilic acid and CSA in biological fluids. In this method, the components are separated by TLC, eluted and quantitated spectrofluorometrically. Schafer et al. (1977) reported a simple procedure for plasma samples in which the components are separated by TLC, followed by quantitation by a direct thin-layer fluorometric method. Steiness et al. (1979) reported a very sensitive method for CSA in both plasma and urine samples and the improved sensitivity is due to a color reaction of CSA with Erlich's reagent.

A number of HPC procedures are published in the literature for the analysis of furosemide. In some of the HPLC procedures described for furosemide, CSA was identified as well. However, in many cases, CSA may not be quantitated under the same conditions, as CSA eluted very fast and usually with the solvent front or components of injection. In the procedure described by Ghanekar et al. (1978) the decomposition products (CSA and others) were co-eluted with the solvent in the stability studies of furosemide dosage forms. In the HPLC procedures of Blair et al. (1975) and MacDougall et al. (1975) a baseline separation of furosemide and CSA was not observed. Roseboom and Sorel reported a HPLC procedure for the analysis of furosemide, in which CSA was shown not to interfere in the analysis of furosemide and similarly Carr et al. (1971) reported the analysis of furosemide, where CSA was clearly separated. However, the applicability of this method for the analysis of CSA was not demonstrated. Recently Smith et al. (1980) reported a procedure for the quantitative analysis of CSA in plasma samples. However, this procedure involves a two detector system. As all the reported literature procedures described so far appear to have some drawback or the other, in this report a simple, specific and sensitive procedure is described for the analysis of furosemide from pharmaceutical preparations.

Materials and Methods

Materials

2-Amino-4-chloro-5-sulfamoylanthranilic acid (CSA) and furosemide reference standards were purchased from the United States Pharmacopeia, Rockville, MD. Furosemide bulk powder, 40 mg furosemide tablets (Lasix), and furosemide injections were obtained from Hoechst-Roussel. Furosemide tablets (Lasix) 20 and 80 mg were purchased from District Wholesale. *p*-Aminosalicylic acid was purchased from

Sigma and anthranilic acid was purchased from Fisher Scientific. Acetonitrile was of HPLC grade and was obtained from Burdick and Jackson. All other chemicals were of reagent grade.

Apparatus

The high-performance liquid chromatograph consisted of a Waters Associates flow pump, Model 6000A, a Waters Associates U6K injector, and a Waters Associates UV detector, Model 440 and a Varian 9176 strip chart recorder. The column used was a C_{18} prepacked stainless steel column (3.9 mm × 30 cm) from Waters. A Waters Associates in-line precolumn filter and a Brown Lee Labs precolumn (4.6 mm × 3 cm, RP-18 10 μm) packed with a reverse phase material were used to remove interfering materials from the sample prior to passage onto the analytical column. For fluorescence detection, a Schoeffel Instrument, Model FS970, fluorescence detector was used. For preparative high performance liquid chromatography, a Waters Associates C_{18} column (7.8 mm i.d. × 30 cm) was utilized. Occasionally the UV detector and the fluorescence detector were used in series.

Chromatographic conditions

The mobile phase for the analysis of CSA from tablets and pharmaceutical preparations consisted of acetonitrile and 0.08 M aqueous phosphoric acid in a ratio of 10:90. A mobile phase with acetonitrile:0.08 M aqueous phosphoric acid, in a ratio of 17:83, was also used in the analysis of purity of the USP supplied CSA. This mobile phase was similar to that of Smith et al. (1980). A mobile phase with acetonitrile and 0.08 M aqueous phosphatic acid, 20:80, was also used in the screening of a number of chemicals for their use as internal standards. The flow rate was 2 ml/min.

Chromatographic detection system

The eluates were detected by either UV or fluorescence spectroscopy. In case of UV detection, the wavelength was set at 280 nm and for detection by fluorescence spectroscopy, the wavelength of excitation was 233 nm and the emission filter used was a 389 nm cut-off filter.

Procedure for the analysis CSA

Twenty-five ml of 0.1 N sodium hydroxide was added to the sample for analysis and the mixture was stirred for 5 min with a magnetic stirring bar. The contents were centrifuged for 5 min at 3000 rpm. A 2-ml aliquot of the clear supernatant was mixed with 100 μl of a solution of the internal standard, anthranilic acid in acetonitrile (1.15 mg/ml). 25 μl of this solution was injected onto the HPLC column.

In the analysis of a single tablet for CSA, the entire tablet was processed for analysis. In this way, 10 individual furosemide 40 mg tablets were analyzed. Composite samples for tablets were obtained by pulverizing 20 tablets and an aliquot of the powder equivalent to a single tablet was processed. Composite powders from 20, 40 and 80 mg furosemide Lasix tablets were prepared and

analyzed. CSA was analyzed in two lots of bulk powders. In the analysis of bulk powder, a sample of approximately 50 mg was processed.

In the analysis of injection, to 1.0 ml of the solution was added 5 ml of 0.1 N sodium hydroxide solution and the solutions were mixed. From this solution, 2.0 ml was pipetted out and to this solution was added 100 μl of 1.15 ng/ml of anthranilic acid in acetonitrile. The contents were vigorously mixed. From this solution, 25 μl was injected onto the HPLC. By this procedure, 6 individual ampoules were analyzed and a pooled composite sample of all the ampoules was analyzed as well in quadruplicate.

Precision

Precision for the analytical method was established by analyzing standard solutions of CSA. All the samples were analyzed in triplicate.

Recovery

Standard solutions with known amounts of CSA were prepared in duplicate. One set was diluted and injected onto the column. A straight-line correlation was obtained when peak heights were plotted as a function of concentration. Similarly, peak heights obtained from experimentally processed samples gave a straight-line correlation when plotted against concentrations. The ratio of the slopes was used to calculate the percent recovery. The recovery was quantitative.

Results and Discussion

A number of procedures are available in literature for the analysis of CSA. However, to our knowledge, none of the procedures have been adapted for the quantitative estimation of CSA from table formulations. The TLC procedures are specific; however, they are cumbersome (Mikkelsen and Andreasen, 1977, Schafer et al., 1977, Steiners et al., 1979, Yakatan et al.). The GLC procedure (Perez et al., 1979) requires derivatization and it is time consuming. Two HPLC procedures (Blair et al., 1975; MacDougall et al., 1975) do not show a base line separation of CSA and furosemide. In another HPLC procedure (Ghanekar et al., 1978), CSA co-elutes with the solvent front or other components of the formulations. A sensitive, simple HPLC procedure was recently reported by Smith et al. (1980). However, the procedure consists of a two detector system, a UV absorption detector to monitor acetanilide (internal standard) and a fluorescence detector for monitoring CSA.

Initially, attempts were made from this laboratory to analyze CSA and furosemide simultaneously using a gradient elution system (Roth et al., 1981). However, attempts to simultaneously analyze the two components using a single internal standard were given up as the two components are present in vastly different amounts and moreover prolonged washing of the HPLC column was required after the use of gradient elution to get reproducible peak heights and to ensure equilibration of the initial column conditions. Hence it seemed advantageous to analyze the components separately. The initial procedure for the preparation of the sample is

similar to that of furosemide (Roth et al., 1981). A portion of the extracted sample is used for the analysis of furosemide and another portion for CSA. A number of compounds were screened as to their suitability as internal standards and it was found that anthranilic acid (Fig. 1) and *p*-aminosalicylic acid appeared to be suitable compounds for use as internal standards. Anthranilic acid was used as the internal standard in all the further studies.

The procedure for isolation of CSA from tablets consisted of vigorous stirring of the sample with 0.1 N sodium hydroxide. As CSA has both an amino and carboxyl function, an organic solvent may not insure a quantitative recovery. However, a strongly basic aqueous solution ensures complete extraction and as expected from our recovery studies, the recovery was quantitative.

The detection system consisted of measuring absorbances at 280 nm. Although the limit test for CSA is 0.4% in furosemide powder, sharp peaks of sufficient peak height to enable accurate measurement (Fig. 2A) are obtained by this method and even at 1/6 the percentage of the limit test, i.e. approximately 0.067%, the procedure could be successfully utilized under the conditions of this procedure (see Fig. 2B). Quantitative estimations well below this percentage could be achieved by modifications of the conditions, i.e. size of the sample, volume of injection, attenuation etc. In Fig. 2A and B are shown the chromatograms, CSA shown at about 0.4% and about 1/6 of that amount. In Fig. 2C the chromatogram of a tablet analyzed for CSA is presented and CSA present is well below the 0.4% limit.

The assay procedure is simple in that it involves addition of internal standard, a simple extraction and injection of the sample onto HPLC. The procedure is precise and average coefficient of variation over the concentration range is around 2% (see Table I). Although the USP limit test for CSA is recommended for composite powder from a number of tablets, this procedure developed could be applied to individual tablets and results on 10 individual determinations of 40 mg furosemide tablets are presented in Table 2. The USP requires that the composite powder from tablets be assayed and that the composite powder from tablets should not contain

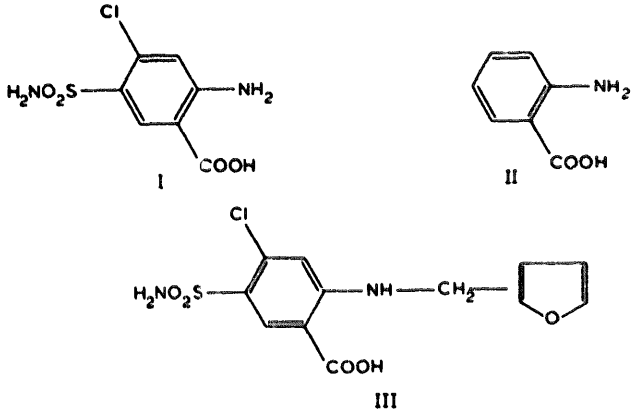

Fig. 1. Chemical structures of 4-chloro-5-sulfamoylanthranilic acid (I, also referred to as CSA), O-amino benzoic acid (II, also referred to as anthranilic acid) and 4-chloro-N-furfuryl-5-sulfamoylanthranilic acid (III, also referred to as furosemide).

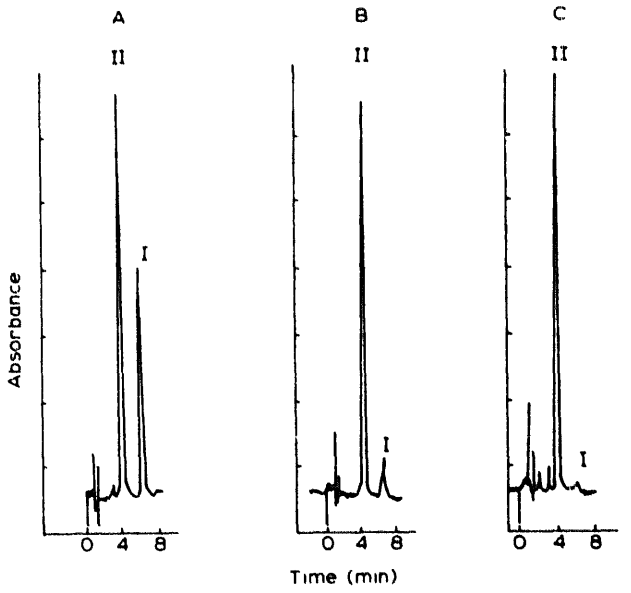

Fig. 2. HPLC chromatogram of a processed sample containing standard mixtures of I and III. I was added to III at a weight percent of 0.4% in A, 0.067 (1/6 the percent as in Fig. A) by weight percent in B. Compound II was the internal standard. C is the HPLC chromatogram as determined by processing an entire tablet of a 40 mg furosemide tablet as described in the procedure.

more than 0.8% CSA. To demonstrate further the suitability of the procedure for the limit tests, CSA was analyzed from tablet composites of 20, 40 and 80 mg tablets of furosemide and at each dosage level, the analysis was done in triplicate. As shown in Table 3, all the tablet composites pass the USP limit test for CSA.

The USP has a limit for CSA in furosemide bulk powder of 0.4%. Using the above procedure, two lots of bulk powders of furosemide were analyzed and the results obtained were 0.05% and 0.02% by weight of furosemide. Both the lots tested contain lower than 0.4% of CSA and the amounts of CSA present is lower than the limit permitted by USP.

The USP limit tests for CSA in furosemide bulk powder, tablets and the injection

TABLE I

ESTIMATION OF PRECISION FOR THE HPLC METHOD FOR STANDARD SOLUTIONS OF FUROSEMIDE

Theoretical amount (ng)	Mean [a]	Range	Coefficient of variation (%)	Relative error (%)
111	111	(105–115)	4.8	-
222	222	(220–226)	1.5	-
333	332	(331–333)	0.4	0.3
444	444	(434–451)	2.0	-

[a] n = 3.

TABLE 2

QUANTITATIVE ESTIMATION OF CSA IN INDIVIDUAL TABLETS

Sample number (mg)	Amount of CSA present/tablet	% by weight of furosemide [a]
1	0.017	0.04
2	0.012 [b]	0.03
3	0.011 [b]	0.03
4	0.011 [b]	0.03
5	0.015	0.04
6	0.011 [b]	0.03
7	0.011 [b]	0.03
8	0.019	0.05
9	0.015	0.04
10	0.015	0.04

[a] 0.8% of CSA in furosemide is the maximum allowed by USP.

[b] Amount of CSA present in the tablet is very low and is below the levels that could be accurately quantitated by the assay.

are 0.4%, 0.8% and 1.0% respectively. The limit test for tablets and injection permit higher limits of CSA compared to the bulk powder, to allow for possible decomposition due to processing and storage for tablets and allow for possible decomposition due to storage under aqueous conditions for the injection. Furosemide injection USP was analyzed for CSA. Six individual ampoules and a composite solution were

TABLE 3

QUANTITATIVE ESTIMATION OF CSA PRESENT IN AN ALIQUOT OF THE COMPOSITE REPRESENTING A SINGLE TABLET

Aliquot of the composite powder representing a tablet	Amount of CSA present/composite	% by weight of furosemide [a]
20	0.009 [b]	0.05
	0.014	0.07
	0.013 [b]	0.07
40	0.013 [b]	0.03
	0.012	0.03
	0.018	0.05
80	0.043	0.05
	0.042	0.05
	0.053	0.07

[a] 0.8% of CSA in furosemide is the maximum allowed by USP.

[b] Amount of CSA present in the tablet is very low and is below the levels that could be accurately quantitated by the assay.

analyzed. The composite solution was made by mixing equal volumes from the 6 ampoules and was analyzed as well. All the 6 individual ampoules pass the USP limit test (Table 4). The average values for CSA from the 6 ampoules is 0.058 ± 0.0098% and the average of the 4 analyses of the pooled injection is 0.062 ± 0.007%. The results are in reasonable agreement.

Thus the procedure developed was demonstrated to be useful for analysis of individual tablets, tablet composites, bulk powders and injectables. The procedure is simple and does not utilize any experimental conditions that result in breakdown of furosemide to CSA. The USP procedure, in addition to being non-specific has certain drawbacks. In the analysis of CSA by the USP procedure, the diazotization step involves utilization of strongly acidic conditions which might result in possible degradation of furosemide to CSA. Furthermore, after diazotization, a colored complex is formed and the absorbances must be measured promptly, since it was found that the intensity of the compounds decreases with time (Table 5).

The purity of CSA standard obtained from USP was in question under UV detection. To elucidate the nature of the impurity, attempts were made to use a fluorescent detecting system as well. The CSA obtained from USP showed a major UV peak and a trace impurity (approximately 2–3% of the peak height area, Fig. 3A); the same USP reference standard, under the same experimental conditions, showed two major fluorescent peaks (Fig. 3B). The faster eluting peak represented approximately 30% of the peak height area and this highly fluorescent, fast eluting peak corresponds in its retention time to the minor peak seen in UV, (compound IV in Fig. 3A and B) suggesting that this compound is highly fluorescent. In order to establish the identity of the two peaks, furosemide USP reference standard was incubated in the presence of 5 N aqueous hydrochloric acid for 4 h at room temperature. HPLC analysis showed the presence of a single degradation product and this degradation product had a retention time identical to the larger fluorescent

TABLE 4

QUANTITATIVE ESTIMATION OF % CSA IN FUROSEMIDE INJECTION

Ampoule no.	% by weight of CSA present [a]	
1	0.049	Ave. = 0.058 ± 0.009
2	0.051	
3	0.073	
4	0.058	
5	0.065	
6	0.049	
Pooled composite		
1	0.073	Ave. = 0.063 ± 0.007
2	0.065	
3	0.056	
4	0.058	

[a] 1.0% CSA in furosemide is maximum allowed by USP.

TABLE 5

STABILITY OF THE COLORED COMPLEX FOR THE USP LIMIT TEST

Time (min)	Absorbance	Absorbance
0	0.168	100
5	0.162	96.4
15	0.156	92.9
30	0.128	76.2
60	0.087	51.8

(slower eluting) peak (Fig. 3C). Hence this larger peak was identified as the degradation product, CSA. The identity of the small peak is not known at the present time. It appears that the faster eluting compound is a highly fluorescent impurity. Although Smith et al. (1980) analyzed CSA from plasma and urine, it is difficult to judge from their chromatograms whether their reference standard CSA showed two peaks or not, as the chromatograms presented appear along with other components of plasma and urine. In order to confirm the presence of this highly fluorescent impurity, two more new reference standards were purchased from USP (however, the same lot no. was present on all the 3 bottles obtained) and an analysis by HPLC showed the presence of the same two fluorescent peaks as before.

In order to quantitate the impurity present in USP reference standard CSA, pure

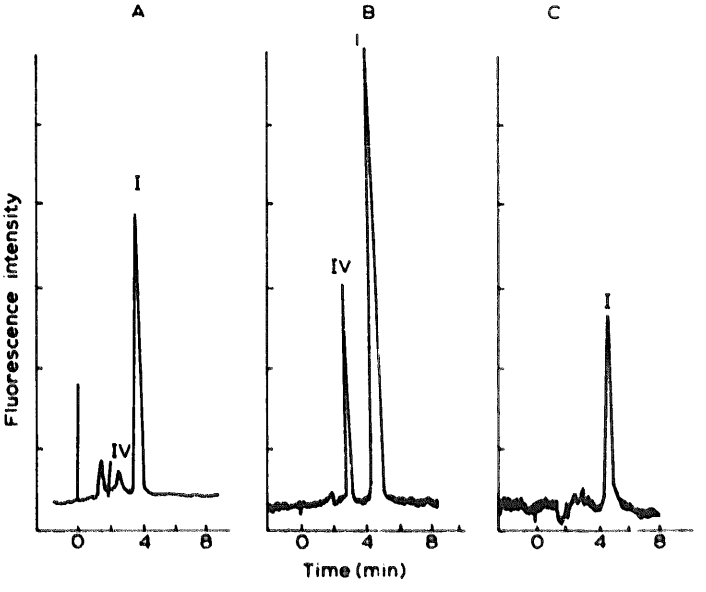

Fig. 3. A: HPLC chromatogram of I USP reference standard, with UV detection. Compound IV is the impurity. B: HPLC chromatogram as in A, with fluorescent detection. C: HPLC chromatogram obtained by treating furosemide USP reference standard (III) with concentrated hydrochloric acid to establish the identify of CSA.

CSA standard is necessary. To obtain pure CSA, a preparative HPLC purification was attempted. A product that showed a single fluorescent peak corresponding to CSA was obtained after two preparative HPLC purifications. The product obtained was slightly hygroscopic and it was not determined if this material has any solvent of crystallization. Using this purified standard, work is currently under progress to assess the percent purity of CSA in the USP reference standard.

Conclusion

A simple, sensitive and specific HPLC analytical method was developed. This procedure might be used to determine the amount of CSA present in furosemide powder, furosemide tablet and furosemide injection. The USP reference standard, CSA, is not pure and shown to contain a highly fluorescent impurity. The USP reference standard, CSA, must be purified if a very sensitive procedure with fluorescence detection is desired.

Acknowledgment

The authors express their sincere gratitude to Dr. T.J. Goehl for his expert advice during the course of this work.

References

Blair, A.D., Forrey, A.W., Meijsen, B.T. and Cutler, R.E., Assay of flucytosine and furosemide by high pressure liquid chromatography. J. Pharm. Sci., 54 (1975) 1334–1339.

Carr, K., Rane, A. and Frolich, J.C.A., Simplified assay of furosemide in plasma and urine by high-pressure liquid chromatography. J. Chromatogr., 145 (1978) 421–427.

Ghanekar, A.G., Das Gupta, V. and Gibbs, C.W., Stability of furosemide in aqueous systems. J. Pharm. Sci., 67 (1978) 808–811.

Haussler, A. and Hasdu, P., Investigations of the saluretic furosemide II. 4-Chloro-N(furfurylmethyl)-5-sulfamoyl-anthranilic acid. Arzneim. Forsch., 6 (1964) 710–713.

MacDougall, M.L., Shoemar, D.W. and Azarnoff, D.L., Separation and quantitative analysis of furosemide and 4-chloro-5-sulfamoylanthranilic acid (CSA) by high pressure liquid chromatography. Res. Commun. Chem. Path. Pharmacol., 10 (1975) 285–292.

Mikkelsen, E. and Andreasen, F., Simultaneous determination of furosemide and two of its possible metabolites in biological fluids. Acta Pharmacol. Toxicol., 41 (1977) 254–262.

Perez, J., Sitar, D.S. and Ogilvie, R.I., Biotransformation of furosemide in patients with acute pulmonary edema. Drug Metab. Disp., 7 (1979) 383–387.

Roseboom, H. and Sorel, R.H.A., Rapid and sensitive determination of furosemide in human plasma by high pressure liquid chromatography. Anal. Lett., B11 (1978) 469–475.

Roth, J., Rapaka, R.S. and Prasad, V.K., An HPLC procedure for the analysis of furosemide in pharmaceuticals—analysis of furosemide tablets and furosemide injection. Anal. Lett., 14 (1981) in press.

Schafer, M., Geissler, H.E. and Mutschler, E., Fluorimetrische Bestimmung Von Furosemid Und 4-chloro-5-sulfamoylanthranilsaure in Plasma Durch Direkte Auswertung Von Dunnschicht Chromatogrammen. J. Chromatogr., 143 (1977) 636–639.

Smith, D.E., Lin, E.T. and Benet, L.Z., Absorption and disposition of furosemide in healthy volunteers, measured with a metabolite-specific assay. Drug Metab. Disp., 8 (1980) 337–342.

Steiness, I.B., Christansen, J. and Steiness, E., Direct thin-layer densitometric determination of pharmacological concentrations of furosemide in plasma and urine. J. Chromatogr., 164 (1979) 241–246.

United States Pharmacopeia XX (1980) 344–345.

Yakatan, G.J., Maness, D.D., Scholler, S., Novick, Wm.J., Jr. and Doluisio, J.T., Absorption, distribution, metabolism and excretion of furosemide in dogs and monkey I: Analytical methodology, metabolism and urinary excretion. J. Pharm. Sci., 65 (1976) 1456–1460.