Journal of Chromatography, 230 (1982) 253—261
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1252

A METHOD FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SCREENING OF UV-POSITIVE COMPONENTS IN URINE ELUATE FROM SEPHADEX G-10 AND MODIFICATIONS FOR DETERMINATION OF URINARY SALICYLIC, SALICYLURIC AND GENTISIC ACIDS

NERMIN FORNSTEDT

Institute of Biochemistry, Biomedical Center, University of Uppsala, Box 576, S-751 23 Uppsala (Sweden)

(First received November 10th, 1981; revised manuscript received February 9th, 1982)

SUMMARY

Acidified urine is chromatographed on a small Sephadex G-10 column, and UV-absorbing urine constituents in an appropriate eluate are screened by reversed-phase high-performance liquid chromatography. Chromatograms of morning-urine specimens from healthy adults show 14—19 peaks. The elution positions of eighteen common aromatic urine compounds have been established. Modifications for the determination of urinary salicylic, salicyluric and gentisic acids are also presented.

INTRODUCTION

The combination of aromatic adsorption chromatography on Sephadex gel and reversed-phase high-performance liquid chromatography (HPLC) with UV absorbance detection provides a simple tool for the determination of some aromatic urine metabolites [1—3]. In order to explore the possibilities of its application to determine some other aromatic urine components, the combination using Sephadex G-10 [2,3] is modified for screening some low-molecular-weight aromatic urine constituents and drugs and drug metabolites. For identification purposes, as well as for developing separate determination methods, the retention properties of 25 aromatic reference substances on G-10 and on a reversed-phase column were established. Methods for the measurement of urinary salicylic acid (SA), salicyluric acid (SUA) and gentisic acid (GA) are presented in this report. A preliminary mass spectrometric investigation of an unknown urine component (see x_4 in ref. 3) is also included in the paper.

0378-4347/82/0000—0000/$02.75

EXPERIMENTAL

Apparatus

The equipment and the operation conditions for the Sephadex G-10 chromatographic isolation and the HPLC—UV measurements previously described [3] were utilized, except that the isolation columns had the dimensions of 6 cm × 4 mm I.D. and 4 cm × 4 mm I.D., respectively, for SA and GA and the detection wavelengths were 300, 300 and 320 nm, respectively, for SA, SUA and GA.

Chemicals and reagents

(1) 0.1 *M* ammonium formate buffer, pH 3. (2) Mobile phases: (A) (for monitoring reference substances and for screening) 200 ml acetonitrile—800 ml glass-distilled and degassed water –0.4 ml concentrated sulphuric acid—100 mg sodium lauryl sulphate (SLS), pH 2; (B) (for SA assay) 200 ml aceto nitrile—200 ml glass-distilled and degassed water—0.4 ml concentrated formic acid, pH 3; and (C) (for a purification approach and for SUA and GA assays) 120 ml acetonitrile –280 ml glass-distilled and degassed water—0.4 ml concentrated formic acid, pH 3. (3) Reference substances (Sigma, St. Louis, MO, U.S.A.) were as listed in Table I. Check [1—3] the accuracy of the respective stock solution, 100 μg/ml distilled water, of SA, SUA and GA. Store the stock solutions at 5°C (stable for one month). Prepare the working standards as in ref. 3 and make also a 43.75 μg/ml standard from the 50 and 37.5 μg/ml GA standards. (4) Pack the isolation columns with Sephadex G-10 (Pharmacia, Uppsala, Sweden), equilibrate and store as in ref. 3.

Procedure

Sample preparation. Use freshly voided morning urine [2,3] for the screening and purification purposes and untimed urine (acidify and centrifuge as for the morning urine) for the determination of aspirin metabolites.

Screening of UV-positive components in protein-free urine. The procedure for the determination of HVA in urine, described in ref. 3, was used with some modifications. Place a set of one to ten equilibrated G-10 columns (12 cm × 4 mm I.D.) over one to ten sets of three 15-ml test tubes, graduated for 10 ml. Run 200 μl of the urine specimen as described. Discard the first fraction (3 ml). Pool the second and the third fractions (10 ml each) and freeze-dry. Dissolve the residue in 0.5 ml of the buffer, centrifuge and inject 20 μl of the supernatant onto the reversed-phase column (for checking of the HPLC—UV system, see ref. 3) and screen for 30 min.

Purification of the component corresponding to peak No. 16 in Fig. 1 (x_4 in ref. 3) from urine. Chromatograph (see calibration process in ref. 3) 200 μl of the specimen on the equilibrated G-10 column (12 cm × 4 mm I.D.) and establish the elution range of component 16, recognizable by its retention characteristics (V_e/V_t = 4.40—4.66 on G-10; t_R on ODS-2 is 20 min in mobile phase A and 7.1 min in C). For isolation and enrichment on an analytical scale, use the following technique. Place two sets of ten columns over 20 sets of two 15-ml

test tubes, graduated for 10 ml, and run a 200-μl specimen on each column as described [3]. Discard the first 5-ml fractions. Keep the second 3.5-ml fractions (a typical chromatogram for the analysis of No. 16 is illustrated in Fig. 2A), pool and freeze-dry. Dissolve the residue in 200 μl of the mobile phase, centrifuge and inject ten times 20 μl of the supernatant onto the ODS-2 column and collect the eluate containing component 16 (see the markings in Fig. 2B), pool and freeze-dry. Dissolve the residue in about 50 μl of the mobile phase, centrifuge and check the spectrometric purity of the component on HPLC–UV (Fig. 2C).

For a mass spectrometric measurement, repeat the described procedure (avoid use of plastic materials all through) at least four times after regenerating the columns overnight.

Determination of SA, SUA and GA in urine. Run 200 μl of the specimen on the appropriate isolation column as described under Determination of HVA in urine in ref. 3. Discard the first fraction of 2.5, 5.0 and 2.5 ml and keep the second fraction of 4.5, 4.0 and 4.0 ml for the SA, SUA and GA assay, respectively, for further separation and quantitative determination on the HPLC—UV system (for checking of HPLC—UV, see ref. 3). Obtain the concentration by means of the respective calibration curve (peak height in mm versus concentration in μg/ml) constructed using working standards run in the same way as the samples.

RESULTS AND DISCUSSION

As can be seen in G-10 chromatograms of 24-h urine specimens [2], many UV-positive urine components are strongly adsorbed by elution with 0.1 *M* ammonium formate buffer, pH 3. Hence, the eluate from G-10 containing low-molecular-weight components in urine was subjected to screening by HPLC—UV. Freshly voided morning urine was utilized throughout the study in order to avoid interference from dietary metabolites and the risk of inadequate collection and of decomposition during storing associated with 24-h urine. The compounds eluting from G-10 at between 2.1 and 15.3 bed volumes (V_t = 1.5 ml) could be resolved into 14—19 peaks on ODS-2 with the chosen conditions. Specimens from healthy adults (n = 15) screened by the present technique, showed chromatographic patterns similar to those illustrated in Fig. 1.

For tentative identification of the peaks, 25 reference substances, mostly aromatic acids and their hydroxyl derivatives frequently found in urine, were checked on G-10 and on ODS-2 columns separately, and their chromatographic properties compared with those of the above-mentioned peaks. A list of the retention data is given in Table I. As can be seen in the table of the retentions on G-10, half of the tested substances showed a V_e/V_t value greater than 4. The affinity of phenolic compounds to Sephadex may be due to interactions between phenolic hydroxyls and the ether oxygens of the crosslinks [4]. Accordingly, the masking of a phenolic hydroxyl by an alkyl group results in a considerably lower retention (Table I). On the other hand, the higher adsorp-

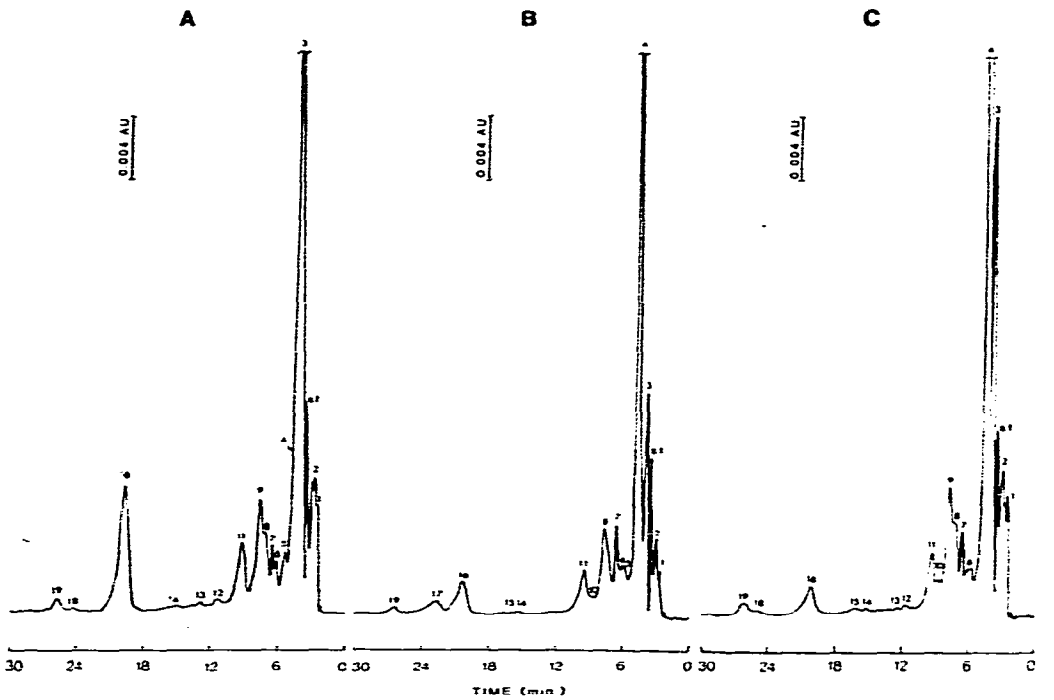

Fig. 1. Typical chromatographic patterns of the HPLC—UV screening of UV-positive components in protein-free urine. Morning urine specimens were obtained from the healthy subjects: (A) female, age 52, weight 55 kg, urine volume 320 ml; (B) male, age 64, weight 79 kg, urine volume 325 ml; (C) female, age 20, weight 58 kg, urine volume 400 ml.

tion of IAA and 5-HIAA, already reported [2], may be caused by a high π-electron-donating ability of such indolic compounds; the presence of uncharacterized π-electron-acceptor groups in Sephadex was suggested earlier [5]. Furthermore, the aromatic acids with unsaturated α—β linkages also adsorb strongly on G-10, as can be seen in the table of the retention values of cinnamic acid and its hydroxyl derivatives. On the other hand, the retentions on ODS-2 seem to be mainly due to reversed-phase interactions. (None of the reference compounds showed any ion pairing with SLS.) Thus, the 2-hydroxy-substituted aromatic acids were retained much more than the 4-hydroxy-substituted ones. By means of the retention values for the reference compounds which elute from the G-10 column within 15 bed volumes and from the ODS-2 column within 30 min, the elution position of several common urine compounds could be determined in the screening diagram. Thus, the position of UA coincides with peak 1, of 3,4-DHMA with peak 2, of 3-HMA, 4-HMA, 3,4-DHPAA, 4-HLA and HGA with peak 3, of 4-HPAA with peak 7, of 3-HPAA, HVA, HA, 4-HBA and GA with peak 8 and of IAA with peak 17. Additionally, SUA and caffeine appear close to peak 12 and ASA close to peak 16. The position of CA is at t_R = 27 min. Although most of the peaks correspond to more than one component, some of them may correspond to a single urine component only. Regarding peak 16, the purification process on an

TABLE I

RETENTION BEHAVIOUR OF REFERENCE COMPOUNDS, RUN ON SEPHADEX G-10 AND ON ODS-2 SEPARATELY

A 200-μl volume of freshly prepared reference solution (100—200 μg/ml) was chromatographed [3] on a G-10 column (12 cm × 4 mm I.D.)* and 10—50 fractions of 0.5 ml monitored** by HPLC—UV (conditions as described in Experimental). t_R values were obtained by direct injections from the respective reference solution.

Compound	V_e/V_t on G-10	t_R (min) on ODS-2
Creatinine	0.73— 1.00	6.0
1,3,7-Trimethylxantine (caffeine)	0.73— 1.00	10.8
2,6,8-Trioxypurine (uric acid, UA)	1.40— 1.66	2.3
Benzoylaminoacetic acid (hippuric acid, HA)	1.40— 1.66	6.9
2-Hydroxyhippuric acid (salicyluric acid, SUA)	4.40— 4.66	11.1
4-Hydroxyphenyllactic acid (4-HLA)	2.06— 2.33	4.3
2-Hydroxybenzoic acid (salicylic acid, SA)	7.06— 7.33	34.0
4-Hydroxybenzoic acid (4-HBA)	10.40—10.66	6.8
2-Acetoxybenzoic acid (acetylsalicylic acid, ASA)	2.06— 2.33	19.3
2,5-Dihydroxybenzoic acid (gentisic acid, GA)	10.73—11.00	6.9
3,4-Dihydroxybenzoic acid (3,4-DHBA)	10.73—11.00	4.3
3-Hydroxymandelic acid (3-HMA)	2.06— 2.33	3.7
4-Hydroxymandelic acid (4-HMA)	2.40— 2.66	3.5
3-Methoxy-4-hydroxymandelic acid (VMA)	1.73— 2.00	3.3
3,4-Dihydroxymandelic acid (3,4-DHMA)	2.73— 3.00	2.6
3-Hydroxyphenylacetic acid (3-HPAA)	3.73— 4.00	7.1
4-Hydroxyphenylacetic acid (4-HPAA)	3.73— 4.00	6.3
3-Methoxy-4-hydroxyphenylacetic acid (HVA)	3.40— 3.66	6.8
2,5-Dihydroxyphenylacetic acid (2,5-DHPAA, HGA)	4.73— 5.00	3.5
3,4-Dihydroxyphenylacetic acid (3,4-DHPAA, DOPAC)	4.73— 5.00	4.0
Indole-3-acetic acid (IAA)	15.77—16.33	22.3
5-Hydroxyindole-3-acetic acid (5-HIAA)	19.20—20.00	5.6
Cinnamic acid (CA)	10.06—10.33	27.0
4-Hydroxycinnamic acid (4-HCA)	23.20—24.00	12.9
3,4-Dihydroxycinnamic acid (3,4-DHCA, caffeic acid)	34.20—36.00	6.8

*G-10 chromatography of 5-HIAA, 4-HCA and 3,4-DHCA carried out on 4 cm × 4 mm I.D. columns.
**Creatinine monitored at 240 nm (not detectable at 280 nm).

analytical scale resulted in a spectrometrically pure isolated substance (Fig. 2C). Furthermore, the compound in question eluted as a single peak in a number of mobile phases with different acetonitrile/water ratios (unpublished results). A preliminary mass spectrometric investigation resulted in the fragmentation patterns illustrated in Fig. 3. Base ions appear at *m/z* 161 (Fig. 3A) and 44 (Fig. 3B). Prominent ions were the fragments at *m/z* 132, 147 and 186 (Fig. 3A) and at *m/z* 28, 29 and 46 (Fig. 3B).

Certainly, gas chromatography (GC) combined with mass spectrometry (MS) is recognized as the main technique for screening and identifying constituents present in human urine (publications until 1977 are reviewed in ref. 6). However, GC is restricted to volatile substances and compounds con-

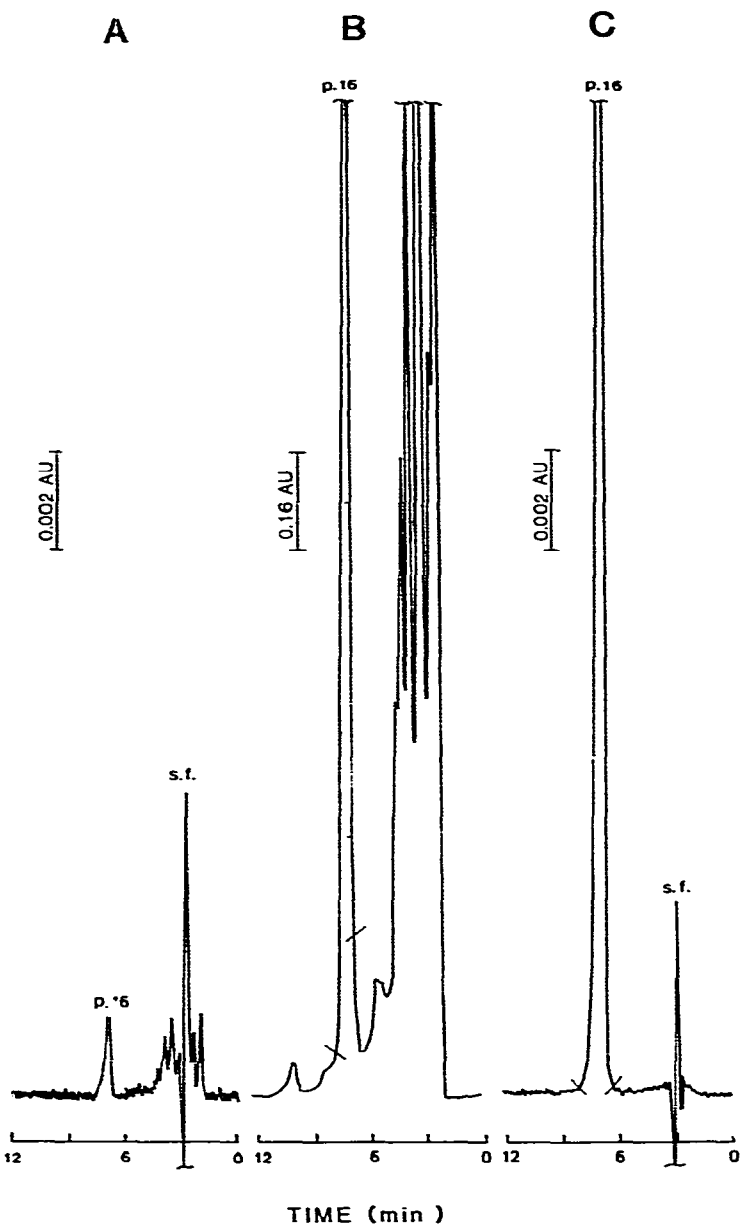

Fig. 2. HPLC diagrams for checking the isolation and enrichment stages for component 16 in Fig. 1 from urine (conditions as in Experimental).

vertible into volatile derivatives and is not suitable for screening high-molecular-weight and/or temperature-labile constituents. Consequently, the HPLC technique constitutes a powerful complement to GC [7,8] by its rapidity, simplicity, reproducibility and efficiency. The simple HPLC—UV technique worked out in this study may be used for preliminary checking of alterations in urinary phenolic acid status, since the formation of methyl esters of such aromatic

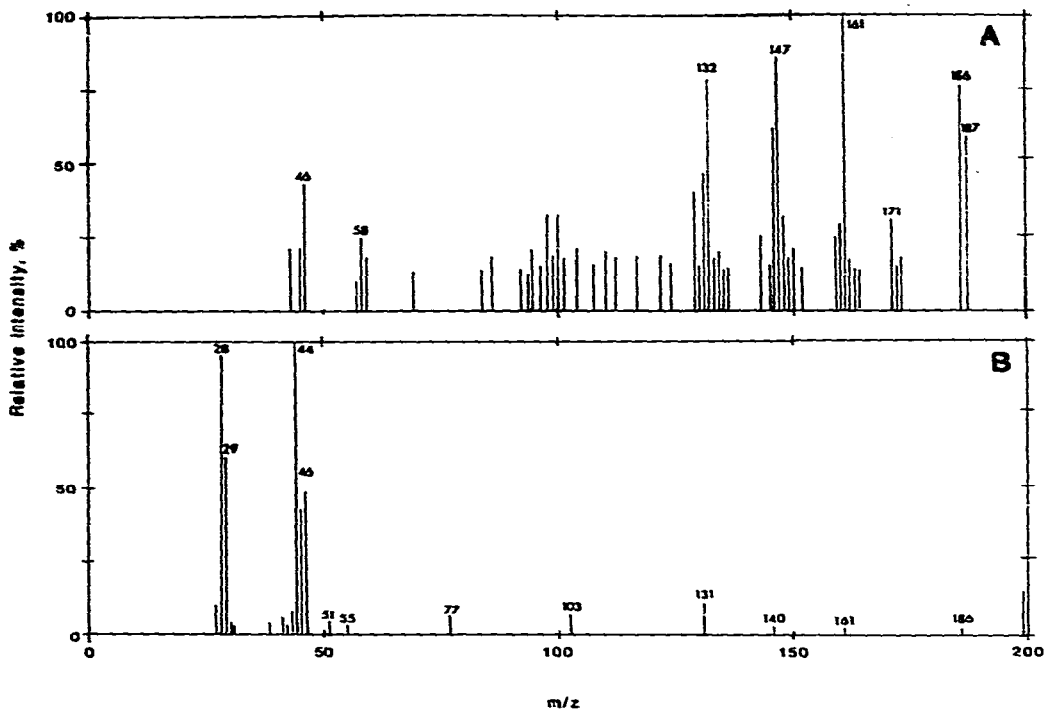

Fig. 3. The 12-eV electron-impact (A) and the 70-eV electron-impact (B) spectra of urine compound 16, obtained on an LKB-9000 mass spectrometer by scanning the region *m/z* 20—400. Operating conditions were: direct inlet temperature, 90°C; ion source temperature, 290°C; trap current, 60 μA.

acids for multiple-component analysis by GC also results in the formation of methyl ethers leading to multiple derivatives [9].

Retention data in Table I were also of use in selecting and developing some new two-step chromatographic determination methods. Typical chromatograms for the determination of some aspirin metabolites are given in Fig. 4. From a dose of one aspirin (about 10 mg of aspirin per kg body weight) 1.1% was excreted after 8 h as SA, 39.2% as SUA and 0.9% as GA. The calibration curves of SA, SUA and GA, which pass through the origin on extrapolation, are linear from 3.125 to, respectively, 50, 37.5 and 43.75 μg/ml. Higher concentrations require dilution of the urine samples. For quantities less than about 2 μg/ml the second fractions will be concentrated [2]. The lowest detectable amounts of SA, SUA and GA in urine are, respectively, 0.5, 0.5 and 0.25 μg/ml, which correspond to 10, 10 and 5 ng injected. The recoveries of added (25 μg/ml urine) SA, SUA and GA were found to be, respectively, 98.7% (0.4% R.S.D., $n = 5$), 96.1% (0.5% R.S.D., $n = 5$) and 99.1% (0.5% R.S.D., $n = 5$).

Furthermore, methods for the measurement of urinary cinnamic acid and its hydroxyl derivatives are possible by small modifications of the described determination methods.

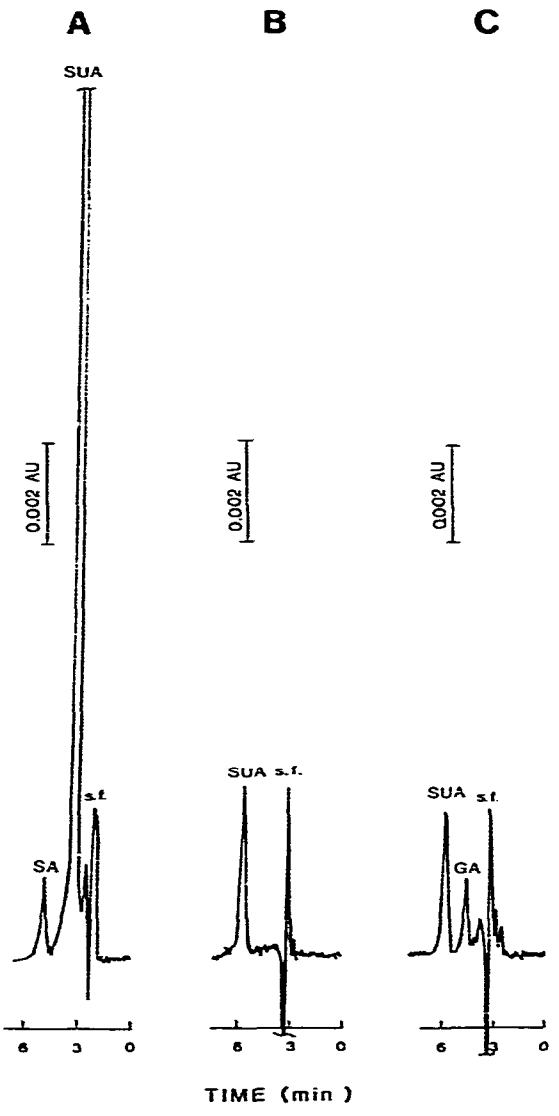

Fig. 4. Typical HPLC diagrams of (A) SA, (B) SUA and (C) GA in urine obtained by the described methods. Concentrations in urine, 8 h after an oral dose of one aspirin tablet (500 mg ASA), were 20.7, 702.0 and 16.4 μg/ml, respectively (voided urine volume = 280 ml; SUA analysis on 20-fold diluted urine).

ACKNOWLEDGEMENTS

The author is indebted to Mr. Peter Jahnke, Research Engineer at the Mass Spectrometry Laboratory of the Biomedical Center, University of Uppsala, for performing the mass spectra of compound 16.

REFERENCES

1 N. Fornstedt, Anal. Chem., 50 (1978) 1342.
2 N. Fornstedt, J. Chromatogr., 181 (1980) 456.
3 N. Fornstedt, J. Chromatogr., 225 (1981) 440.
4 A.J.W. Brook and K.C. Munday, J. Chromatogr., 47 (1970) 1.
5 A. Sada, G. di Pascale and M.G. Cacace, J. Chromatogr., 177 (1979) 353.
6 E. Jellum, J. Chromatogr., 143 (1977) 427.
7 I. Molnár and Cs. Horváth, J. Chromatogr., 143 (1977) 391.
8 F. Geeraerts, L. Schimpfessel and R. Crokaert, J. Chromatogr., 145 (1978) 63.
9 G.K. Brown, O. Stokke and E. Jellum, J. Chromatogr., 145 (1978) 177.