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Note

Liquid chromatographic determination of drugs in urine by direct injection on to a reversed-phase column

Fluorescence versus UV detection

PER-OLOF LAGERSTRÖM
Department of Analytical Chemistry, AB Hässle, S-431 83 Mölndal (Sweden)

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In the routine analysis of drugs in biological samples by liquid chromatography (LC) and spectroscopic detection the sample work-up procedure is often the most time-consuming step. Direct injection of the samples without any prior purification (extraction) will of course simplify the analyses and shorten the analysis time. However, the following prerequisites generally must be valid. (1) The compound possesses such properties that it can be selectively detected, such as absorbance at a wavelength where few other compounds absorb or high fluorescence. (2) The concentration of the compound in the sample is relatively high, because in most cases only a relatively small sample volume can be injected (20—50 μl). (3) The column can withstand injection of a large number of samples without any degeneration; otherwise there is little to gain compared to a regular work-up procedure.

In biopharmaceutical studies for the development of more efficient drug preparations there is a need to measure the amount of drug excreted in urine during a certain time interval. Compounds that we have analysed in connection with biopharmaceutical studies by direct injection of urine samples on to columns with a chemically bonded non-polar phase are quinidine (dihydroquinidine), furosemide, salicylic, salicyluric and gentisic acid, salicylazosulfapyridine and N-acetyl-5-aminosalicylic acid.

During the last few years simplified methods for drug determination, mostly in plasma but also in urine, have become increasingly popular. After protein precipitation, plasma samples have been injected direct on to an LC column. Methods for analysis of quinidine [1], furosemide [2—4], salicylic acid [5] and metabolites [6] and N-acetyl-4-aminosalicylic acid [7] in plasma have been published. Furosemide [3, 4] and salicylic acid and metabolites [8] have also

been determined in urine samples. Fluorescence [1, 2, 4, 7] and UV detection [3, 5, 6, 8] were used.

In this study UV and fluorescence detection have been compared for sensitivity and selectivity. The signal-to-noise ratio for fluorescent compounds is often not dependent on the type of detector (UV or fluorescence), while the selectivity in most cases is much better with the fluorescence detector, which increases the overall sensitivity of the analytical procedure.

EXPERIMENTAL

Chromatographic apparatus

The liquid chromatograph consisted of an Altex 110A (Berkeley, CA, U.S.A.) pump, an LDC spectromonitor III (Riviera Beach, FL, U.S.A.) UV detector and a Perkin Elmer LC 1000 (Norwalk, CT, U.S.A.) fluorescence detector. The injector was from Rheodyne (Berkeley, CA, U.S.A.; 70-10) with a 20-μl loop. The separation column of stainless steel (length 150 mm, O.D. 6.35 mm, I.D. 4.5 mm) had end fittings of modified Swagelok® connections. Operations were carried out at room temperature.

Chemicals and packing material

Acetonitrile, dichloromethane and hexane (pro analysi; E. Merck, Darmstadt, G.F.R.) were used.

Tetrabutylammonium hydrogen sulphate ($TBAHSO_4$) obtained from the Department of Organic Chemistry, AB Haessle, Mölndal, Sweden, was neutralized with sodium hydroxide to pH 5—10 and purified by shaking three times with dichloromethane (one-tenth volume) and two times with hexane.

All reagent and buffer solutions were prepared with analytical-reagent grade chemicals.

The drug compounds fulfilled the quality requirements of the Pharmacopoeia Nordica.

The packing materials used in the separation columns were LiChrosorb RP-8 (average diameter 5 or 10 μm) and RP-18 (5 μm) (E. Merck). The performance of the columns was maintained by exchange of the particles at the top of the column every day.

Analytical procedure

The general scheme for the analytical procedures is as follows: The thawed urine samples are shaken and centrifuged. (Filtration is an alternative to centrifugation but there is a risk of adsorption losses in the filter.) The volume injected on to the LC column is 20 μl.

The chromatographic conditions for each compound are summarized in Table I. Chromatograms for each compound are shown in Figs. 1—5. Comparisons are made between detection by UV and fluorescence.

Quantitative evaluation

In the routine analysis of drug levels in urine samples, peak heights were measured and the concentrations were obtained by comparison with analysed standard urine samples. No internal standard was used owing to the very simple procedure.

RESULTS AND DISCUSSION

In the selection of experimental conditions for determination of drug levels in urine by direct injection of the sample, the characteristics of the analytes, for example fluorescence or absorbance at a wavelength where other sample components do not interfere, were utilized. Furosemide, quinidine, salicylic, salicyluric, gentisic and N-acetyl-5-aminosalicylic acid are all fluorescent and are often excreted in relatively high concentrations in urine. By injection of 20 μl of authentic samples on to a reversed-phase column, using acetonitrile—buffer mixtures as mobile phases, each of these six compounds could easily be separated from other urine components and detected with a fluorescence detector (Figs. 1—4). Furosemide, being a diuretic drug and thus producing a rather diluted urine sample, could also be selectively detected with a UV detector (Fig. 1). This was not possible for the others (Figs. 2—4) since other UV-absorbing compounds interfered in the chromatograms. Salicylazosulfapyridine is non-fluorescent but absorbs at a rather high wavelength (360 nm, Fig. 5), a valuable property in terms of selectivity.

Since a large number of unpurified urine samples are injected, the column will sooner or later degenerate. Column performance will be maintained over a longer period, however, if the bonded silica particles at the top of the column are exchanged daily or if an exchangeable guard column is used.

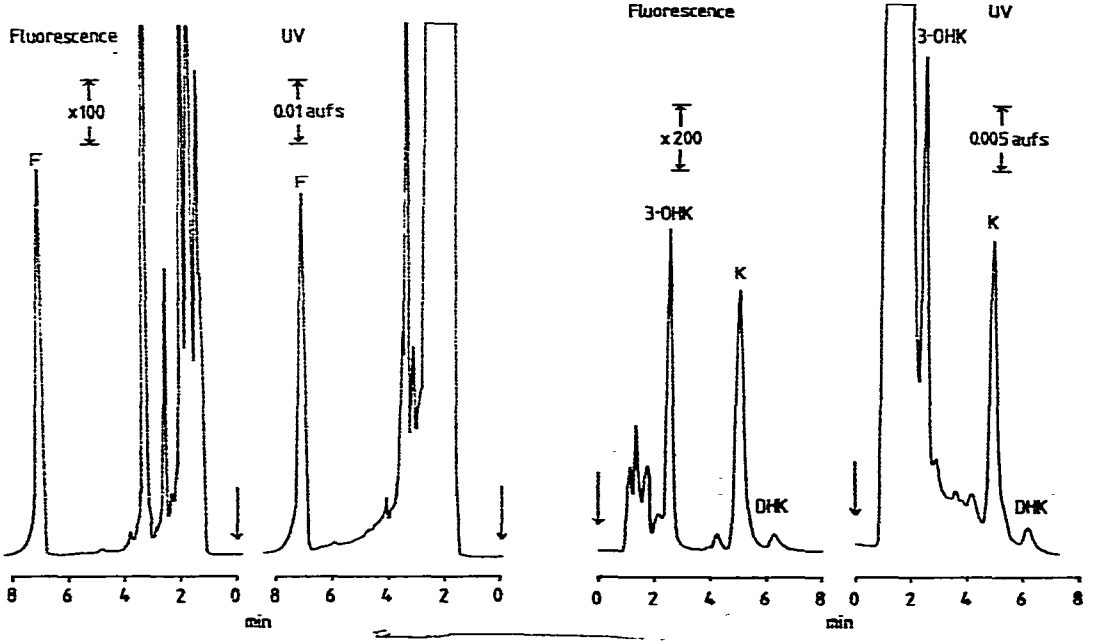

Fig. 1. Furosemide (F) in 20 μl of an authentic urine sample containing 20 μmol/l furosemide injected directly on to an LC column. Detection by UV and fluorescence.

Fig. 2. Quinidine in 20 μl of an authentic urine sample containing 40 μmol/l quinidine (K), 3 μmol/l dihydroquinidine (DHK) and 25 μmol/l 3-hydroxiquinidine (3-OHK) injected directly on to an LC column. Detection by UV and fluorescence.

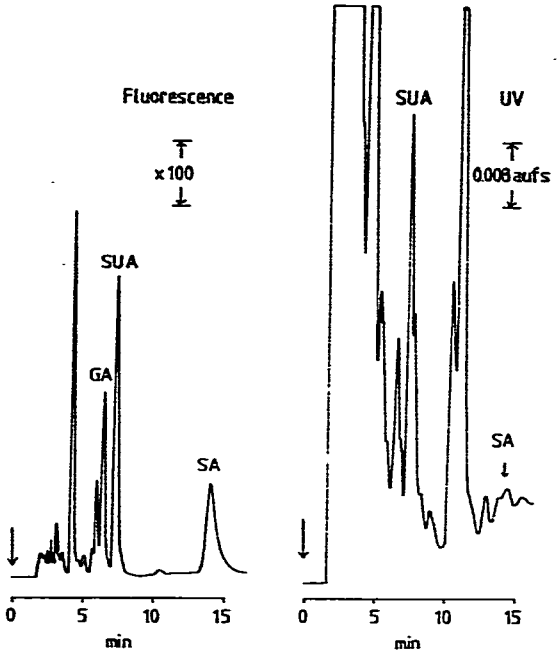

Fig. 3. Salicylic (SA), salicyluric (SUA) and gentisic (GA) acid in 20 μl of an authentic urine sample containing 80 μmol/l SA, 400 μmol/l SUA and 60 μmol/l GA injected directly on to an LC column.

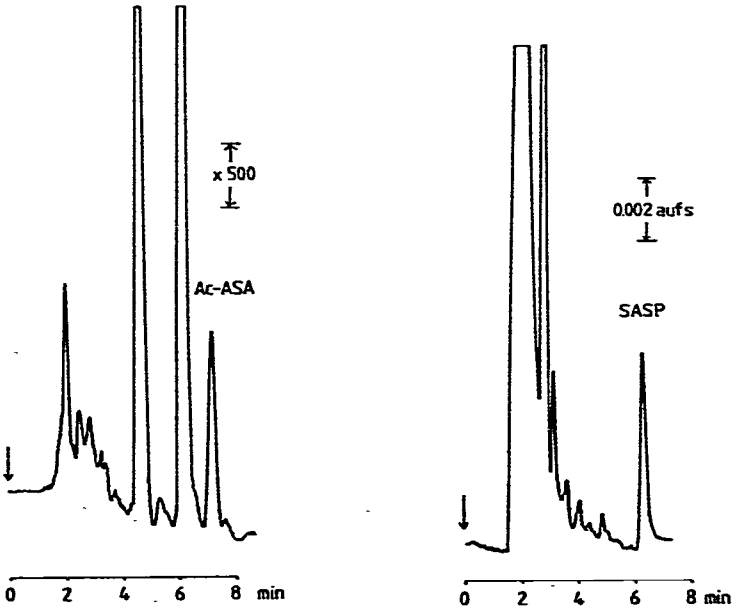

Fig. 4. N-Acetyl-5-aminosalicylic acid (Ac-ASA) in 20 μl of an authentic urine sample containing 8 μmol/l N-acetyl-5-aminosalicylic acid injected directly on to an LC column, with fluorescence detection.

Fig. 5. Salicylazosulfapyridine (SASP) in 20 μl of an authentic urine sample containing 3 μmol/l of salicylazosulfapyridine injected directly on to an LC-column, with photometric detection.

TABLE I

ANALYTICAL PROCEDURES AND CHROMATOGRAPHIC CONDITIONS

Sample: urine. pH of sample is not adjusted. Injection volume: 20 μl. Column: LiChrosorb RP-8, 150 × 4.5 mm.

Drug	Aqueous mobile phase	Flow-rate (ml/min)	Wavelength (nm)		MDC* (μmol/l)	Chromatogram shown in Fig. No.
Furosemide	Acetonitrile 33% H_3PO_4 0.1 mol/l	1.0	270/>389 274	(F)** (UV)	0.2 (UV, F)	1
Quinidine (dihydroquinidine	Acetonitrile 25% $NaClO_4$ + $HClO_4$ (0.095 + 0.005 mol/l)	1.4	363/440 254	(F) (UV)	0.3 (UV, F)	2
Salicylic acid, salicyluric acid, gentisic acid	Acetonitrile 22% TBA 0.01 mol/l phosphate buffer (pH 7, I = 0.05)	1.0	315/420 280	(F) (UV)	5—10	3
N-Acetyl-5-aminosalicylic acid	Acetonitrile 20% TBA 0.01 mol/l phosphate buffer (pH 6.5, I = 0.075)	1.0	315/430	(F)	1	4
Salicylazosulfapyridine***	Acetonitrile 22.5% phosphate buffer (pH 7.6, I = 0.007)	1.0	360	(UV)	0.2	5

*Minimum determinable concentration, S.D. ⩽ 10% (n = 10).
**F = fluorescence.
***Column: LiChrosorb RP-18.

The minimum determinable concentration for each compound is given in Table I, and is defined as the concentration giving a relative standard deviation of ⩽ 10% (n = 10). At concentration levels higher than 5—10 times the minimum determinable concentration the relative standard deviation for each compound was ⩽ 2%.

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