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THE USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR SAMPLE CLEAN-UP IN MASS FRAGMENTOGRAPHIC ASSAYS

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SUMMARY

A novel high-performance liquid chromatography (HPLC) sample clean-up procedure for use in mass fragmentographic assays of (sub)-nanogram amounts of drugs in human plasma is described and compared with a conventional extraction sequence for sample purification. With the assay of the new antidepressant drug mianserin hydrochloride (Org GB 94) as an example, the HPLC procedure is discussed with respect to retention time, recovery, purification, column deterioration and convenience. It is demonstrated that HPLC sample clean-up is a useful and time-saving procedure for routine clinical analyses.

INTRODUCTION

Because of its sensitivity and specificity, mass fragmentography [1] is often used as a detection technique in assays of (sub)-nanogram amounts of drugs in biological fluids. Notwithstanding the specificity that is possible, direct low-resolution mass fragmentographic (LRMF) analyses of crude plasma extracts are impossible in many instances because of the presence of interfering substances originating from the plasma, the solvents and the reagents or the glassware used. Therefore, many ultrasensitive LRMF assays include a sample clean-up procedure to eliminate these interfering substances. For basic drugs, the clean-up of a crude extract is usually performed by extraction into an acidic solvent followed by removal of lipids by washing with non-polar solvents. These often tedious and time-consuming procedures are not amenable to automation and the limited number of samples that can be processed daily by one technician does not permit large-scale routine clinical analyses.

Most of the sample clean-up can be avoided by adjusting the mass spectrometer to a resolution of 10,000 or more and performing high-resolution mass fragmentographic (HRMF) measurements [2]. However, HRMF does not allow the simultaneous registration of internal standard ion peaks, resulting in a loss of accuracy and precision. Moreover, any sensitivity gained by elimination of

sample losses during the clean-up procedure is lost because of the reduced sensitivity of the mass spectrometer at high resolution.

The applicability of high-performance liquid chromatography (HPLC) as the sole purification step prior to LRMF has been investigated in order to simplify the time-consuming sample preparation. This paper describes the potential of HPLC for sample clean-up in comparison with a sequential extraction procedure; both methods were applied to the antidepressant drug mianserin hydrochloride (Org GB 94; 1,2,3,4,10,14b-hexahydro-2-methyldibenzo[*c,f*]pyrazino[1,2-*a*]-azepine monohydrochloride). Details of the assay procedure and some applications are described elsewhere [3].

MATERIALS AND METHODS

Standard and marker compounds

Amitriptyline, used as an HPLC marker compound, was kindly supplied by Merck Sharp & Dohme Nederland B.V. (Haarlem, The Netherlands). The internal standard [10,10-$^2H_2$] Org GB 94, for quantification and the HPLC marker compound [^{3}H] Org GB 94 were prepared by base-catalyzed isotope exchange.

Solvents

All solvents were purchased from Merck (Darmstadt, G.F.R.). *n*-Hexane and isopropanol were of Uvasol quality, ethanol and methanol were of analytical-reagent grade and the ammonia was of Suprapur grade.

Equipment

A Waters Assoc. (Milford, Mass., U.S.A.) Model ALC-202 high-performance liquid chromatograph equipped with a 2-ml loop-containing atmospheric pressure injection system (Type U6K) was used in the preparative mode. The chromatograph was equipped with a 30 cm × 4 mm I.D. stainless-steel column filled with μPorasil (10 μm; Waters Assoc.) operated at a flow-rate of ca. 2ml/min at a pressure of about 800 p.s.i. The standard UV detector was operated at 280 nm. The elution system consisted of *n*-hexane—isopropanol (80:20, v/v) to which 4% of ethanol and 0.1% of concentrated ammonia were added. A combined Varian Aerograph 2740 gas chromatograph—Varian-MAT CH7 mass spectrometer system was used. The gas chromatograph was equipped with a 4 m × 2 mm I.D. glass column filled with 1% JXR on Gas-Chrom Q, operated at 260°. The mass spectrometer was set for monitoring the total ion current (TIC) and for dual ion recording at *m/e* 264 and 266, these being the molecular ion peaks of Org GB 94 and the deuterated internal standard, respectively.

HPLC clean-up

Plasma samples of 1-ml volume, to which known amounts of deuterated Org GB 94 are added as internal standard, are extracted with two 5-ml portions *n*-hexane and the combined extracts are evaporated to dryness at 45° under a gentle stream of nitrogen. The residue is re-dissolved in 0.5 ml of the HPLC solvent system containing 1 μg of amitriptyline* and the entire solution is in-

*Once the HPLC retention time has been determined and established to be constant during an appropriate period, the marker compound can be omitted.

jected into the high-performance liquid chromatograph. The column effluent is trapped during the period from ca. 5 min after injection up to to the disappearance of the amitriptyline peak (at ca. 9 min). This solution is evaporated to dryness and the residue is re-dissolved in 8 μl of methanol prior to combined gas chromatography –mass spectrometry (GC—MS) measurements.

Sequential extraction clean-up

For comparison of the extent of purification, a sequential extraction procedure is also used. The crude *n*-hexane extract is re-extracted twince with 1.5 ml of 0.1 *N* hydrochloric acid. The combined acidic layers are washed with two 5-ml portions diethyl ether in order to remove lipids, and the aqueous phase is adjusted to pH 10 and subsequently extracted with *n*-hexane. The purified extract is evaporated to dryness and re-dissolved in 8 μl of methanol prior to GC—MS measurements.

RESULTS AND DISCUSSION

Because the HPLC procedure described here is a novel approach to the clean-up of plasma samples, some aspects are described below in more detail.

Establishment of retention times

The use of microgram amounts of the compound to be measured for establishment of retention times should be avoided as far as possible because of the risk of cross-contamination. The eluate fractions to be trapped should be selected in such a way that appropriate separation from interfering impurities is achieved and that quantitative recoveries of the compound and internal standard are guaranteed. This is achieved either by the use of radioisotope-labelled compounds and measurement of radioactivity in the eluate as a function of time, or by using appropriate marker compounds. Both procedures are illustrated in Fig. 1, which shows the HPLC 280-nm UV detector signal and the amount of radioactivity in subsequently collected 0.5-ml fractions of a plasma sample to which 7 nCi (= 0.1 μg) of titrated Org GB 94 and 1 μg of amitriptyline were added as marker compounds*.

As shown, Org GB 94 is eluted before amitriptyline. By collecting the eluate during the period from 1 min after the impurity peak up to the end of the amitriptyline peak, a quantitative recovery can be expected (see *Recovery*).

Recovery

For nanogram amounts of Org GB 94, the recovery from the HPLC was determined to be almost 100%. For microgram amounts some material is lost in the chromatograph. The carry-over to a subsequent blank injection was found to be of the order of 0.1%, which does not permit alternate processing of samples that contain microgram and nanogram amounts of Org GB 94. This

*While establishing the retention time, one should be aware of a possible separation between the compound to be determined and its deuterated analogue. In some instances, differences in retention times of more than 1 min are observed [4].

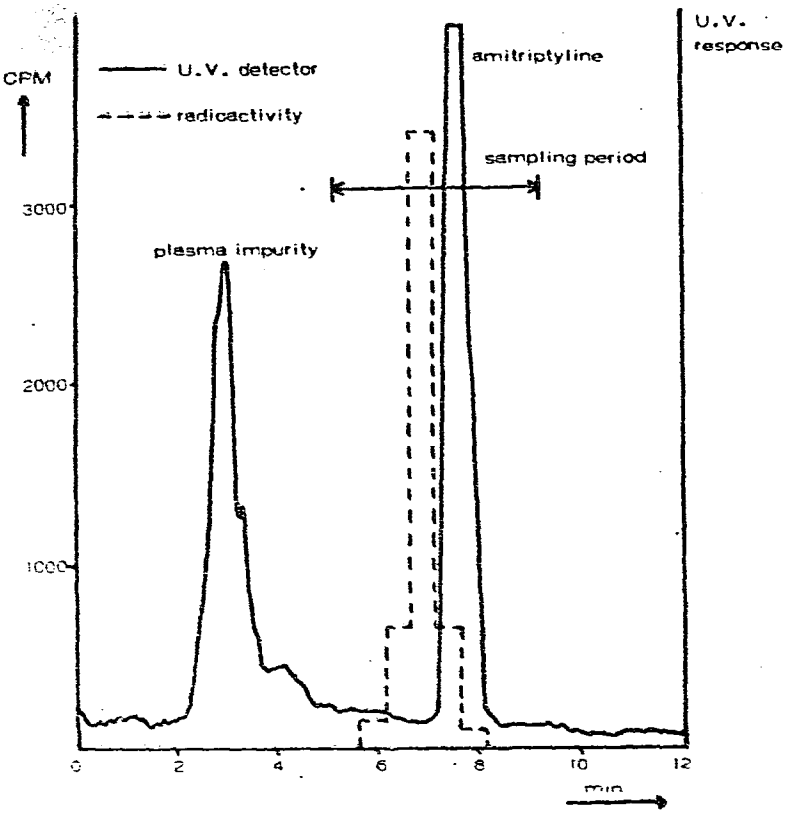

Fig. 1. High-performance liquid chromatogram (280 nm) of a 1-ml plasma extract containing 7 nCi [^{3}H]Org GB 94 (= 0.1 μg) and 2 μg of amitriptyline as marker compounds. The broken line shows the radioactivity in the 0.5-ml eluate fractions collected.

carry-over also illustrates that for the establishment of retention times, microgram amounts of the compound to be determined can not be used. Within a series of nanogram analyses, carry-over of this order of magnitude does not seriously affect the accuracy of the assay.

Extent of purification

The extent of purification is illustrated by comparison with the sequential extraction clean-up method (cf., Materials and Methods) and with the crude extract. The extracts were made from 2-ml blank plasma samples spiked with 4 ng of Org GB 94 and 4 ng of [10,10-$^2H_2$]Org GB 94. The resulting mass fragmentograms and TIC recordings are shown in Figs. 2 and 3, respectively.

The LRMF trace of the crude extract at *m/e* 264 (Fig. 2a) reveals two major impurity peaks (components 1 and 3). The sum of their peak heights exceeds the Org GB 94 peak by a factor of 1.7. In the trace at *m/e* 266, only one major impurity peak (component 2') shows up, while some minor impurities are observed at greater retention times. The TIC trace shows two major impurities with retention times < 2 min and an abundant component with a retention time of 14.3 min.

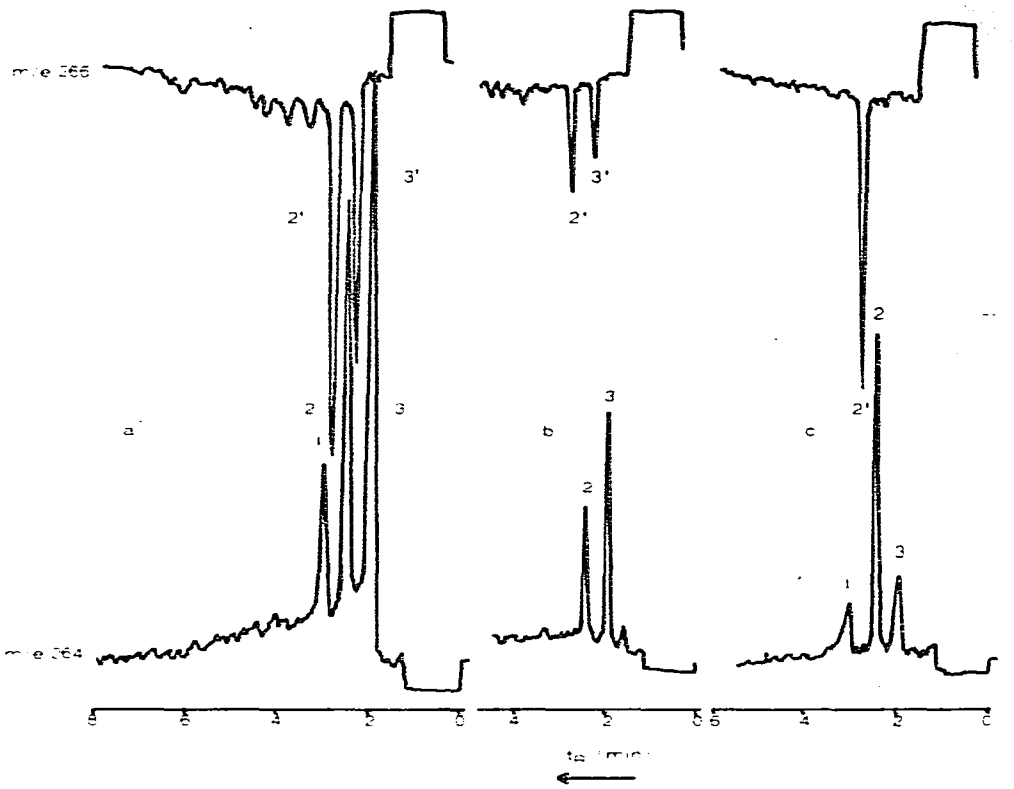

Fig. 2. Mass fragmentograms recorded at *m/e* 264 and *m/e* 266 of 2-ml blank plasma samples spiked with 4 ng of Org GB 94 and 4 ng of [10,10-$^2H_2$]Org GB 94 after (a) a single extraction, (b) a back-extraction clean-up and (c) an HPLC clean-up.

In the LRMF trace after the sequential extraction clean-up method (Fig. 2b), component 1 with *m/e* 264 disappeared, while compounds 3 and 3′ were still present with the same abundance relative to Org GB 94 as in the crude extracts. The ion intensities, however, are smaller by a factor of about 3. The corresponding TIC trace shows that the impurity peaks with short retention times are reduced whereas the component with a retention time of 14.3 min disappeared.

After HPLC purification, the trace at *m/e* 264 (Fig. 2c) shows only small residues of compounds 1 and 3, while in the trace at *m/e* 266 no other impurities show up. The Org GB 94 peak height is ca. 2.5 and 0.8 times the peak height after sequential extraction clean-up and single extraction, respectively. The corresponding TIC trace again shows further purification.

Column deterioration

With the μPorasil column currently in use, we processed over 2000 plasma samples. By flushing the column once every 100—200 samples with appropriate polar or acidic solvents, its performance could be maintained.

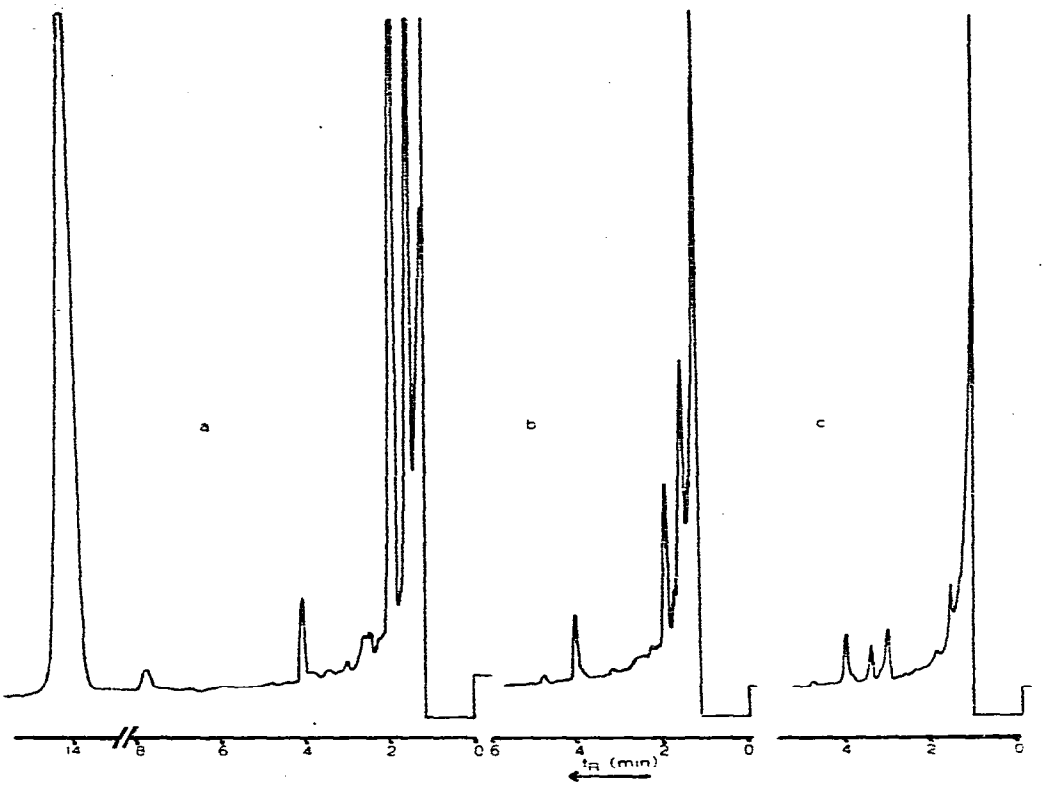

Fig. 3. TIC recordings of 2-ml blank plasma samples spiked with 4 ng of Org GB 94 and 4 ng of [10,10-$^2H_2$]Org GB 94 after (a) a single extraction, (b) a back-extraction clean-up and (c) an HPLC clean-up.

Convenience and speed

Because of its simplicity, the HPLC clean-up step can be performed by unexperienced technicians. Purification of one crude plasma extract takes 5—10 min, depending on the flow-rate used in HPLC and on the compound to be measured. An average of 60 samples can be processed per day. Because the HPLC procedure is the rate-limiting step, the total analysis capacity is also about 60 samples a day.

In contrast to other purification methods such as repeated extractions or thin-layer chromatography, the HPLC method can be automated relatively simply. This automation will improve the capacity and reliability, which are prerequisites for routine clinical analyses.

CONCLUSION

The HPLC sample clean-up proved to be a reliable, convenient and time-saving procedure for use in routine clinical mass fragmentographic quantifications.

If a small number of samples are to be assayed, a crude *n*-hexane extract can be used. For routine analyses, when 50—60 samples a day are to be processed, the crude extracts will contaminate the gas chromatographic column and mass spectrometer to an unacceptable extent and under these circumstances an HPLC clean-up provides adequate purification without significant loss of sensitivity.

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