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Note

Gas chromatographic analysis of naloxone in biological fluids

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Several methods have been described for the analysis of naloxone in biological fluids [1—5]. However, only the radio-immunoassay of Berkowitz et al. [3] has sufficient sensitivity to enable the characterisation of naloxone disposition in man after usual doses. Sams and Malspeis [5] have described a gas chromatographic method with electron capture detection, based on the formation of a perfluoroalkylester derivative of naloxone which has comparable sensitivity to the method of Berkowitz et al., but this technique has not been applied to biological samples. We describe a method which has equivalent sensitivity and reproducibility to the radio-immunoassay of Berkowitz et al., but offers a chromatographic approach to the analysis of naloxone in biological fluids.

EXPERIMENTAL

Method

The sample preparation is illustrated in Fig. 1. Naltrexone, a close structural analogue of naloxone (Fig. 2), was used as an internal standard. The internal standard solution contained 100 ng of naltrexone per 100 μl of 0.1 *M* sulphuric acid. Each sample was assayed as follows. Whole blood or plasma (1 ml) and 100 ng of internal standard were added to a 100 × 13 mm culture tube with a PTFE-lined screw-cap, which contained 250 μl of carbonate buffer (pH 9, 1.0 *M*), together with 5 ml of toluene. Each tube was mixed by tilting for 30 min on a mechanical mixer and centrifuged at 1000 *g* for 5 min. The organic and aqueous layers were separated by freezing each tube in a dry-ice—acetone bath and pouring the unfrozen organic layer into a second tube containing 250 μl of 0.1 *M* sulphuric acid. This tube was mixed for 5 min, frozen in dry-ice—acetone and the toluene discarded. To the remaining aqueous phase in each

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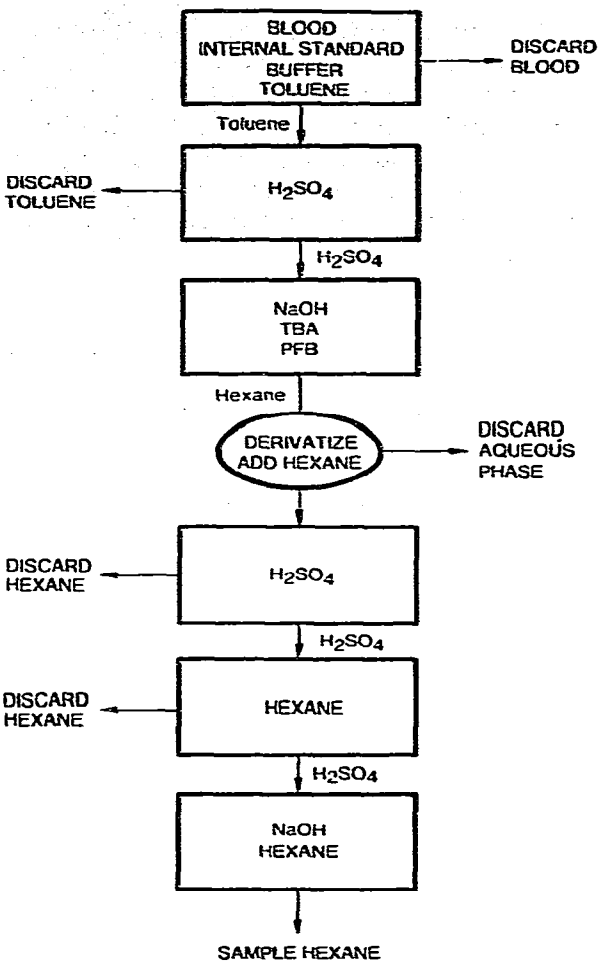

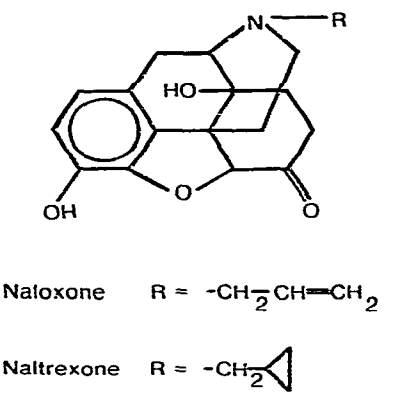

Fig. 1. Flow diagram of sample preparation procedure.

Fig. 2. Structures of naloxone and the internal standard (naltrexone).

tube were added 500 µl of aqueous 0.1 *M* tetrabutylammonium hydroxide (TBA) and 200 µl of a 0.4% solution of pentafluorobenzyl bromide (PFB) in dichloromethane. This mixture was shaken at room temperature for 30 min in order to carry out the derivatisation reaction. A 2-ml volume of hexane was added to each tube, which was then vortexed for 10 sec, centrifuged for 5 min and frozen in dry-ice—acetone.

The organic layer was poured into a third 100 × 13 mm culture tube, containing 0.5 ml of 0.5 *M* sulphuric acid. This mixture was vortexed for 1 min, centrifuged for 1 min and the organic phase discarded. This procedure was repeated with an additional 2 ml of hexane. Finally, 100 µl of 5 *N* sodium hydroxide solution and 100 µl of hexane were added to the tube, which was mixed for 5 min and centrifuged for 5 min. Approximately 5 µl of the hexane layer was sampled from the tube and injected into the gas chromatograph.

Apparatus

A 1.8 m × 3.2 mm glass column packed with 3% OV-17 on Gas-Chrom Q, 100—120 mesh (Applied Science Labs., PA, U.S.A.) was used in a Hewlett-Packard Model 5710A gas chromatograph fitted with a ^{63}Ni electron-capture detector. The column was conditioned at 300°C for 48 h before being connected to the detector. The oven temperature was 280°C, the injector port 250°C and the detector 350°C. The flow-rate of the carrier gas (5% methane in argon) was 60 ml/min. The output from the detector was recorded with a dual-pen recorder with voltage spans set at 1 and 5 mV.

Calibration

The assay was calibrated by analysing samples containing, 1, 5, 10, 25, 50, 100, 150 and 200 ng of naloxone. The peak height ratio of naloxone to naltrexone was plotted versus the amount of naloxone in each sample. Linear and power functions were fitted to these data using least-squares regression analysis. The reproducibility of the assay was measured by determining the coefficient of variation for ten samples, each containing the same amount of naloxone. The reproducibility was determined in this way for 5 and 50 ng of naloxone.

RESULTS AND DISCUSSION

Under the conditions described the retention times were 4.5 min for naloxone and 6.75 min for naltrexone. Fig. 3 shows a chromatogram from a 1-ml control blood sample (A) and from a 1-ml blood sample containing 5 ng of naloxone and 100 ng of internal standard (B). There were no significant peaks which interfered with the peaks of interest in control blood samples from six subjects.

Fig. 4 shows a typical calibration curve for naloxone from whole blood. Although the data shown in Fig. 4 could be reasonably well fitted by the equation for a straight line [$y = 0.103 + 0.0184x$, $r^2 = 0.9091$, where y is the peak height ratio of naloxone to naltrexone, x is the amount of naloxone in the sample and r is the correlation coefficient], close examination shows data have an upward trend. This visual impression was confirmed by dividing the peak height ratio by the amount of naloxone in the sample to give a normalised peak height ratio. When analysed in this manner, the normalised peak height ratio for the 1-ng sample was 1.04 and this value increased progressively with increasing amounts of naloxone, to reach a value of 1.62 for the 200-ng sample. For this reason data in Fig. 3 were fitted to a power function ($y = ax^b$), $y = 0.00856x^{1.11447}$, $r^2 = 0.998$. The mean r^2 value for 12 calibration curves prepared over a period of several months was 0.989 + 0.02 (S.D.).

Attempts to produce linear calibration curves, by reducing on-column loss of the naloxone derivative with the injection of desmethylimipramine as described by Brotell et al. [6] and Swezey et al.[7] for a similar analysis of pentazocine, were not successful.

The coefficient of variation in the peak height ratio of ten blood samples containing the same concentration of naloxone was 2.68% for 50 ng and 4.1% for 5 ng. Calibration curves from plasma were similar to those obtained for whole blood.

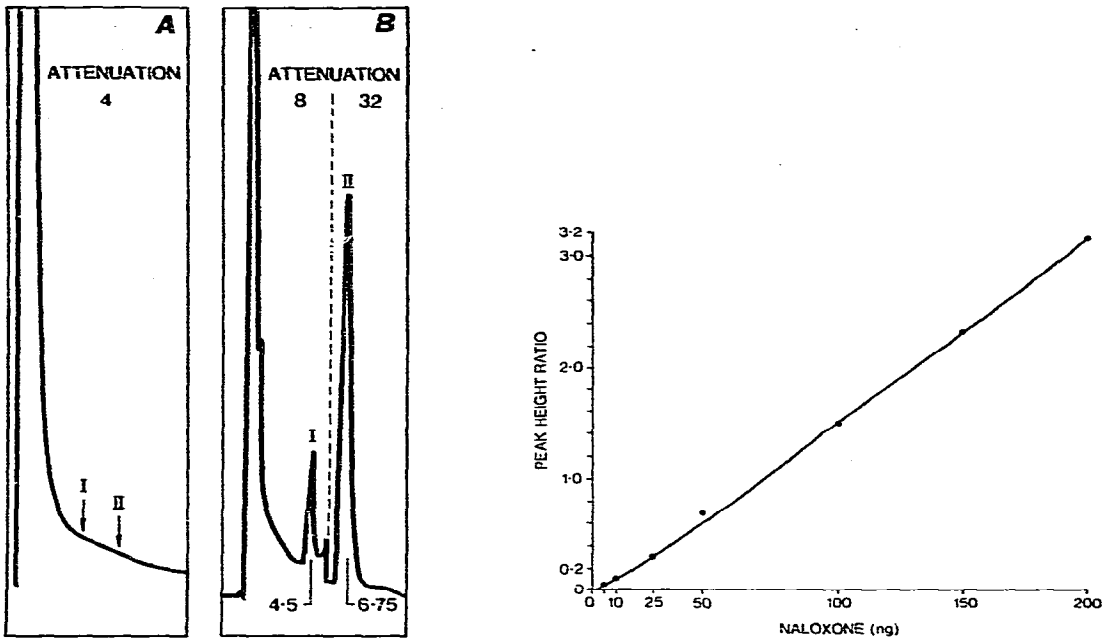

Fig. 3. Chromatograms of (A) control blood sample and (B) sample containing 5 ng of naloxone (I) and 100 ng of naltrexone (internal standard) (II). I and II in A indicate the retention times of naloxone (4.5 min) and internal standard (6.75 min).

Fig. 4. Calibration curve for naloxone. Data were fitted to the expression $y = 0.00856x^{1.11447}$, $r^2 = 0.998$.

It is sometimes convenient when analysing large numbers of samples to prepare samples on one day and chromatograph them the following day. The stability of the derivatised samples was investigated by preparing calibration samples on one day and immediately chromatographing them and then re-chromatographing them 24 h later, during which time the samples had been stored at 4°C. When a power function was fitted to these data, values of $a = 0.0209$, $b = 1.212$, $r^2 = 0.999$, were obtained on the first chromatographic analysis and $a = 0.0233$, $b = 1.182$, $r^2 = 0.998$, were obtained 24 h later. This analysis indicates that during this period storage at 4°C did not affect the accuracy of the method.

Of the previously available methods for the analysis of naloxone in biological fluids, only the radio-immunoassay of Berkowitz et al. for which the lowest point on the calibration curve is 5 ng, has sufficient sensitivity to enable the disposition of naloxone to be determined after usual doses. The analysis of naloxone reported by Sams and Malspeis [5] which utilises gas chromatography with electron capture detection has good sensitivity (2 ng) and reproducibility (coefficient of variation 1—8.5%) but has not been applied to biological samples. A further disadvantage of this method is the instability of the perfluoroalkylester derivatives of naloxone and the internal standard naltrexone, which gives rise to changes in peak height ratio with time. The present analysis was designed to retain the sensitivity of electron capture detection while elim-

inating the disadvantages of the ester derivatives of naloxone. Our approach is based on that described by Swezey et al. [7] for pentazocine, in which a more stable ether derivative is formed by ion-paired extractive alkylation of the phenolic anion using tetrabutylammonium hydroxide and pentafluorobenzyl bromide.

Ether derivates of naloxone and naltrexone formed in this way are stable to strong acid and alkali and thus show no tendency to hydrolyse prior to chromatography as do the ester derivatives described by Sams and Malspeis [5]. The stability of the ether derivatives of naloxone and naltrexone also facilitates the removal of excess derivatising agent prior to chromatography (see Fig. 1).

Although ethyl acetate [2] and 1% isopropanol in chloroform [4] have been used to extract naloxone from biological fluids, preliminary experiments with [^{3}H]naloxone in which 1 ml of blood, adjusted to pH 9, was extracted with 5 ml of organic solvent, showed that, under these conditions, toluene extracted 78% of the radioactivity. Corresponding values for other solvents were hexane 25%, ethyl acetate 78% and diethyl ether 88%. For reasons of convenience, safety and to enhance specificity, toluene was selected as being the most suitable solvent [7].

In summary, the approach described provides a chromatographic method with equivalent sensitivity and reproducibility to the radio-immunoassay method of Berkowitz et al. [3]. Only these two methods offer sufficient sensitivity to enable the characterisation of the disposition of naloxone after usual doses.

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