Journal of Chromatography, 383 (1986) 158—165
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3296

Note

Determination of ketocyclazocine in human plasma by gas chromatography—negative-ion chemical-ionization mass spectrometry

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(First received February 25th, 1986; revised manuscript received July 10th, 1986)

Ketocyclazocine (KC, Fig. 1) is a synthetic benzomorphan derivative which embodies a number of the structural features of morphine including: (a) a benzene nucleus; (b) a hydroxyl group and a quaternary carbon attached to the benzene nucleus; and (c) a tertiary nitrogen two methylene groups removed from the quaternary carbon. Substantial alterations from morphine in the structure of KC include introduction of a keto group at the benzylic carbon and a cyclopropylmethyl group substitution at nitrogen. These combined structural features of KC produce pharmacological effects sufficiently distinct from morphine to lead Martin et al. [1] to propose that KC interacts with a separate and distinguishable receptor (κ-opioid receptor) from that of morphine (μ-opioid receptor).

Fig. 1. Structures of ketocyclazocine (KC) and ethylketocyclazocine (EKC).

Although assays for the related benzomorphan, cyclazocine, have been developed [2, 3], methods for the measurement of KC in plasma were not available for use in pharmacokinetic studies. Since KC is considerably more potent

than morphine, it was anticipated that an assay for the parent drug in the 1—10 ng/ml range would be required for plasma analysis.

This paper describes the developed procedure for the extraction, derivatization and analysis of KC in plasma by gas chromatography—negative-ion chemical-ionization mass spectrometry (GC—NICIMS). For the assay, a homologue of KC, ethylketocyclazocine (EKC, Fig. 1), was used as the internal standard (I.S.). This procedure has been applied to the analysis of plasma samples following single, intramuscularly administered doses of KC in human subjects.

EXPERIMENTAL

Drugs and reagents

KC and EKC were kindly provided by Sterling-Winthrop Research Institute (Rensselaer, NY, U.S.A.). Pentafluoropropionic anhydride (PFPA) was purchased from Pierce (Rockville, IL, U.S.A.). All other chemicals were of reagent-grade quality.

Instrumentation

A Finnigan Model 4021 GC—MS instrument was operated either in the negative-ion or positive-ion chemical-ionization mode. A glass column (91.4 cm × 2 mm I.D.) packed with 3% OV-11 on Gas-Chrom Q (100—120 mesh) was coupled to the mass spectrometer by a glass-lined stainless-steel tube. The injector, column, interface oven and source were maintained isothermally at 210, 205, 250 and 250°C, respectively. Methane was used as carrier and reagent gas at a flow-rate of 50 ml/min. The electron energy was 70 eV.

Glassware

The glassware used in the extraction and derivatization procedures was acid-washed (nitric acid) following routine cleaning. After extensive rinsing with deionized water, the glassware was oven-dried. Immediately prior to their use, centrifuge tubes were rinsed twice with ethyl acetate (6 ml) and the rinse was discarded.

Subjects, dosing, sample collection

The subjects were healthy, drug-free male volunteers with a history of opiate and hallucinogen abuse. During the study the subjects were housed on the clinical ward of the Addiction Research Center (Baltimore, MD, U.S.A.). All gave informed consent to participate in the study, the protocol for which had been approved by the Institutional Review Board of Francis Scott Key Hospital. KC was administered intramuscularly as a single dose (0.6—1.0 mg). Heparinized blood was collected at specified times, centrifuged and the plasma was frozen until time of assay.

Extraction and derivatization

Aliquots (1 ml) of plasma samples were transferred into 13-ml glass centrifuge tubes. I.S. (100 ng) was added, followed by one drop of 10 *M* sodium hydroxide solution and 1 ml of 10 *M* phosphate buffer (pH 8.0) to give

a final pH of 8.0 ± 0.1. The samples were shaken on a counter-top shaker for 30 min, centrifuged, and the organic phase was transferred to a clean 13-ml centrifuge tube. To the organic phase were added 3 ml of 0.05 *M* sulfuric acid. The tubes were vortexed for 30 s and centrifuged. The organic phase was discarded by aspiration and two drops of 10 *M* sodium hydroxide solution were added followed by 1 ml of 10 *M* phosphate buffer (pH 8.0). The tubes were briefly vortexed and the pH was adjusted to 8.0 ± 0.1 if necessary. Ethyl acetate (6.0 ml) was added and the tubes were vortexed for 30 s and centrifuged. The organic phase was transferred to a clean 13-ml centrifuge tube. Acidic methanol [0.5 ml, 1% (v/v) hydrochloric acid in methanol] was added and the extract was evaporated to dryness under a nitrogen stream at 40°C. The residue was dissolved in methanol (0.5 ml) for subsequent derivatization and analysis.

Prior to derivatization the methanolic extracts were evaporated to dryness as previously described. Ethyl acetate (75 μl) and PFPA (25 μl) were added and the tubes were incubated at room temperature for 1 h. After incubation the solution was carefully evaporated to dryness under a stream of nitrogen at room temperature. The residue was dissolved in ethyl acetate (25 μl) for analysis by GC—NICIMS.

Quantitative analysis by GC—NICIMS

Extracts of samples and standards were analyzed by GC—NICIMS mass fragmentography (MF). The ions monitored for KC were *m/z* 147 and 284 at a retention time of 1.88 min and for I.S. were *m/z* 147 and 298 at a retention time of 2.91 min. A daily standard curve was constructed from the analyses of standard solutions of KC and I.S. added to control plasma. Duplicate samples (1 ml) containing KC (0, 1, 2.5, 5, 10, 25 ng) and I.S. (100 ng) were analyzed in the same manner as subject samples. A linear response for the peak intensity ratio of KC-PFPA (*m/z* 147) to I.S. (*m/z* 298 or 147) versus concentration was obtained. The correlation coefficient was typically > 0.98. The lower limit of the assay was ca. 0.1 ng/ml. Within-assay variability for 10 ng/ml KC was 6.2% (n = 6). The calculated mean (± S.E.) concentration of KC was 10.09 (± 0.25) ng/ml.

Area under the concentration curve (AUC) over time for KC was calculated by the trapezoidal rule [4]. Pharmacokinetic parameters were estimated with PCNONLIN (Statistical Consultants, Lexington, KY, U.S.A.) on an IBM personal computer (IBM, Boca Raton, FL, U.S.A.).

RESULTS AND DISCUSSION

An MF assay was developed for KC in human plasma. Samples were extracted with ethyl acetate, back-extracted into acid solution, and re-extracted at pH 8.0 with ethyl acetate. Recoveries of KC from plasma by this method were greater than 85%. Following derivatization with PFPA, an aliquot was analyzed by MF with GC—NICIMS. Methane was used as both carrier and reagent gas. Quantitation was performed by the internal standard method with ethylketocyclazocine as the I.S.

Optimization of recovery of KC from plasma together with elimination of

extraction interferences was necessary in order to achieve the required sensitivity for assay of KC in the low ng/ml concentration range. Although the extraction efficiency of ethyl acetate for the recovery of KC from plasma is greater than 85%, significant adsorption losses occurred when extracts were stored overnight at 0° C. Addition of a small amount of acidic methanol to the extract followed by evaporation under a stream of nitrogen and addition of methanol to the residue prevented adsorption losses. Substantial interferences which were apparent early in assay development were traced to reagent and/or glassware contamination. These were completely eliminated during cleaning by an acid-soak of the glassware and an ethyl acetate rinse immediately prior to use.

Derivatization of KC with PFPA improved the chromatographic characteristics of KC and substantially increased the sensitivity of detection when analyzed by methane GC—NICIMS. Fig. 2 illustrates the detector

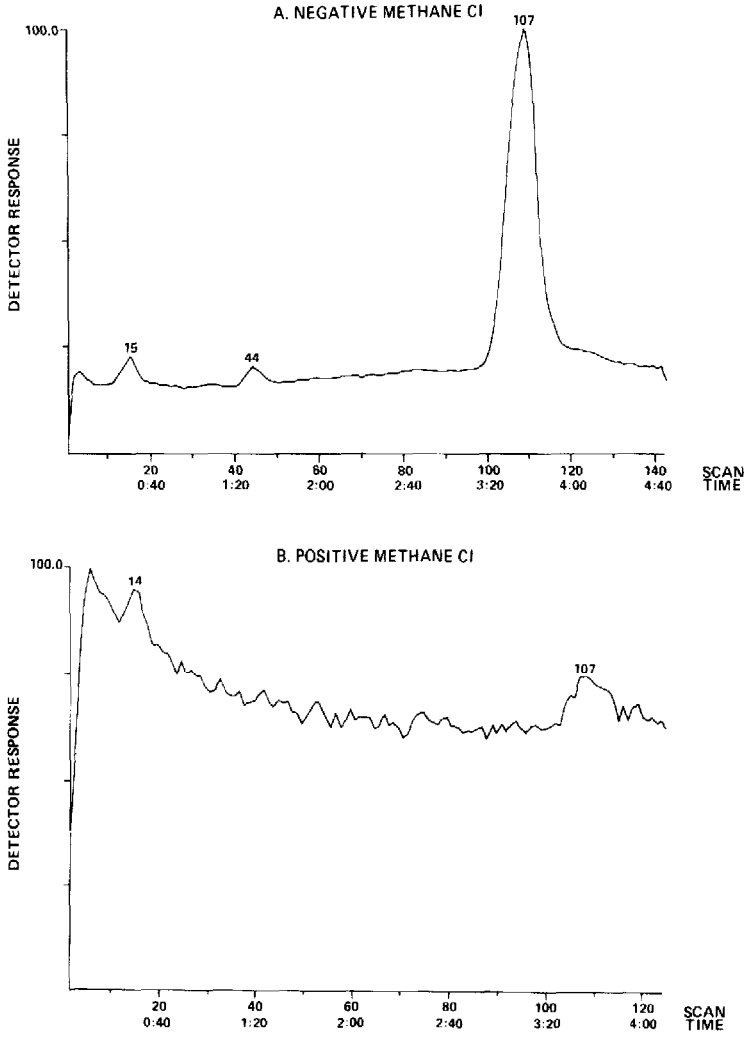

Fig. 2. Total-ion current chromatogram following injection of 10 ng on-column of KC-PFPA. (A) Response under negative methane CI conditions; (B) response under positive methane CI conditions. Samples were run consecutively and all other settings remained the same.

TABLE I

MASS SPECTRA OF KC AND DERIVATIZED KC AND EKC UNDER POSITIVE AND NEGATIVE IONIZATION MODES

Compound	Molecular weight	Scan range	Ionization mode	Spectra (m/z)*				
				[M+29]	[M+1]	M	[M−1]	Prominent fragment ions**
KC	285	60—600	Pos. EI	—	—	285(6)	—	244(6), 214(6), 161(9), 160(7), 159(7), 149(5), 145(8), 131(7), 115(8), 107(5), 105(5), 98(10), 97(85), 96(27), 91(8), 83(12), 82(100), 79(5), 77(10), 70(12), 69(13), 68(26), 67(5), 66(7)
KC-PFPA	431	180—650	Pos. CI	460(13)	432(100)	431(20)	430(15)	314(5), 286(20), 285(15), 284(9), 270(1)
KC-PFPA	431	140—650	Neg. CI	460(0)	432(0)	431(0)	430(0)	284(12), 163(7), 147(100)
EKC-PFPA	445	140—650	Neg. CI	474(0)	446(0)	445(0)	444(0)	298(11), 147(100)

*Percentage abundance in parentheses.

**Only ions ≥ 5% abundance are reported.

response (total-ion current chromatogram) to 10 ng of KC-PFPA on column when analyzed by negative methane CI (panel A) versus positive methane CI (panel B). The samples shown in Fig. 2 were analyzed consecutively with all settings identical with the exception of the mode of ionization. The increase in sensitivity by over a factor of 100 by negative CI results from the ability of KC-PFPA to capture near thermal electrons and the higher rate of negative-ion formation by resonance electron capture over that of ion molecule reactions occurring in positive CI. The rate constant for formation of a negative ion by capture of a low energy electron can be as high as 400 times greater than that for an ion molecule reaction [5].

The pattern of ionization of KC-PFPA by negative methane CI was distinctly different from that produced by positive methane CI (Table I). Most of the positive-ion sample current is carried by the $(M+1)^+$ ion whereas the molecular anion was not observed in the negative-ion spectrum of KC-PFPA or EKC-PFPA. The major ion in the spectrum of KC-PFPA by negative methane CI was *m/z* 147, most likely representing the $(CF_3CF_2CO)^-$ anion fragment. The only other observable ions were *m/z* 163 and 284 which represent $(CF_3CF_2CO_2)^-$ and $(KC - 1)^-$ anion fragments, respectively. The negative methane CI spectrum of EKC-PFPA is consistent with this fragmentation pattern. In contrast to CI, ionization of KC by positive electron impact (EI) produced a plethora of ions with the most abundant ion occurring at *m/z* 82.

For assay of KC-PFPA by MF with GC—NICIMS, the ions at *m/z* 147 and

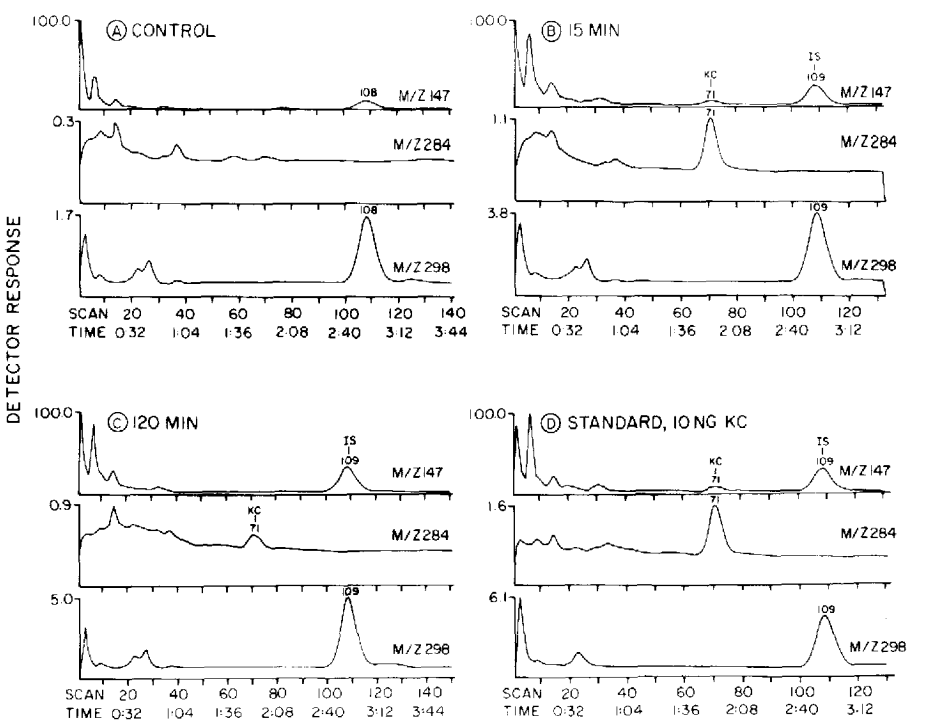

Fig. 3. Mass fragmentograms of plasma extracts with I.S. added (100 ng). (A) Pre-drug control plasma; (B) plasma sample obtained 15 min after a 1.0-mg intramuscular dose of KC; (C) plasma sample as in (B) obtained at 120 min; (D) control plasma with 10 ng KC added. All extracts were derivatized with PFPA and assayed by methane GC—NICIMS.

284 were selected for monitoring and confirmation. The I.S. was monitored by ion *m/z* 298 although the response at the appropriate retention time by *m/z* 147 was equally applicable. The relative response for KC-PFPA at *m/z* 284 to I.S. at *m/z* 298 was 1.3. Plots of peak intensity ratios of KC-PFPA to I.S. were linear across the concentration ranges tested (1—25 ng/ml) with the lower limit of sensitivity being ca. 0.1 ng/ml. Fig. 3 illustrates the MF response for extracted samples from a human subject who received a single 1.0-mg intramuscular dose of KC. As can be seen in Fig. 3A there were no interferences in the pre-drug control sample nor were there interferences in control samples without I.S. At 15 min following drug administration, plasma levels of KC were estimated to be 8.9 ng/ml (Fig. 3B). These levels readily declined and by 120 min were estimated to be 0.7 ng/ml (Fig. 3C). For comparison, the response of an extracted standard sample (10 ng/ml KC) is shown in Fig. 3D.

Mean plasma levels of KC following single intramuscular doses (1.0 mg, n = 3; 0.8 mg, n = 4; 0.6 mg, n = 4) are shown in Fig. 4. Initial plasma levels at 15 min ranged from 6 to 10 ng/ml and declined in a complex, multiexponential fashion to approximately 1 ng/ml in 2—3 h. The mean data from the 1.0- and 0.8-mg drug trials were fitted to a two-compartment model with first-order input with a microcomputer program for the analysis of nonlinear models (PCNONLIN). The mean elimination half-life of KC was estimated to be 45 and 32 min, respectively, for the 0.8- and 1.0-mg doses. Although the kinetic profile of KC in man was complex, the calculated area under the plasma—time curve versus dose appeared to be linear (Fig. 4, inset) over the relatively narrow dose range tested.

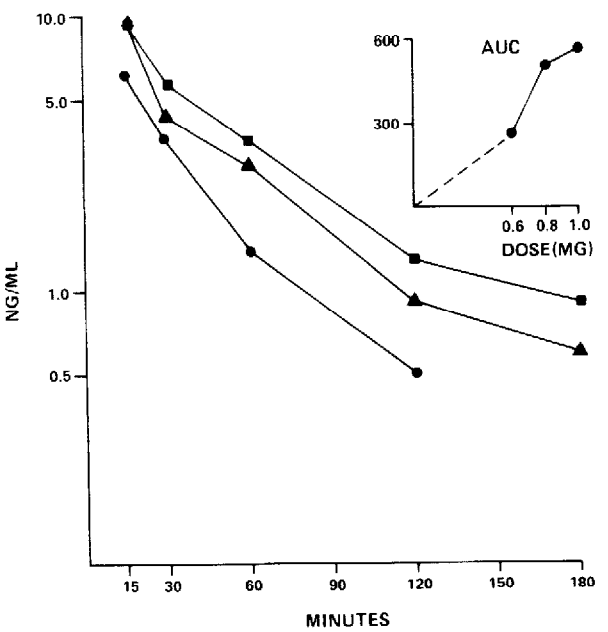

Fig. 4. Mean plasma levels of KC in human subjects following a single intramuscular dose. Mean area under the curve (AUC) for plasma KC versus dose is shown in the inset. The data represent the mean of four subjects for the 0.6-mg (●) and 0.8-mg (▲) doses of KC and the mean of three subjects at the 1.0-mg (■) dose of KC.

Overall, the methodology developed for the assay of KC in plasma proved both specific and sensitive for use in pharmacodynamic studies. In addition, the methodology is readily adaptable for the assay of other benzomorphans including ethylketocyclazocine. This assay should prove useful as more specific, κ-type benzomorphans are developed.

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